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(54) Title: ENHANCED EFFICACY OF LIPOSOMAL ANTISENSE DELIVERY (57) Abstract Pharmaceutical compositions for the treatment of pathologic conditions associated with the overexpression of ICAM-1 in a host. These pharmaceutical compositions comprise an effective amount of an ICAM-1 antisense molecule encapsulated in a lipid mixture which is typically a liposome or lipid particle. The lipid mixture will typically comprise at least two members selected from the group consisting of phospholipids, sterols and cationic lipids.		

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ENHANCED EFFICACY OF LIPOSOMAL ANTISENSE DELIVERY

BACKGROUND OF THE INVENTION

Research aimed at developing therapeutic drugs based on the inhibition of protein expression by oligonucleotides has encountered a number of limitations. It has been demonstrated *in vitro* that a number of cell types exhibit low rates of intracellular delivery of antisense molecules. This is of great concern as antisense molecules are designed to bind to mRNA target sites located in the cytoplasm and nucleus of cells. *In vivo* studies have also indicated that many oligonucleotides are rapidly cleared from circulation; thus a limited time period exists in which the antisense molecule can interact with the target cells. Furthermore, a number of studies have demonstrated that oligonucleotides are quite toxic when administered *in vivo*.

Many of the limitations of free antisense, such as toxicity and nuclease digestion, can potentially be overcome by encapsulating antisense molecules in liposomes. When made as relatively small particles (approximately 100 nm in diameter), liposomes will passively accumulate at sites of inflammation by moving through the restructured vasculature. Numerous studies have determined that liposomes or lipid complexes have the ability to deliver oligonucleotides intracellularly through two mechanisms: cellular uptake of liposomes via endocytosis, and fusion of cationic liposomes with target cell membranes.

Comparisons of free and encapsulated oligonucleotides, indicate an enhanced stability for encapsulated oligos *in vitro* as the liposome prevents nuclease degradation. In contrast, comparison of free and encapsulated phosphorothioate oligonucleotides usually indicate no enhancement as the phosphorothioate oligos are themselves nuclease resistant. Thus the same amount of oligonucleotide can potentially be delivered to the cell whether it is a free phosphorothioate or an encapsulated phosphodiester oligonucleotide. Wang, *et al.*, *Proc. Natl. Acad. Sci.* 92:3318-3322 (1995). Despite such studies, no evidence has been presented which demonstrates that a conventional neutral liposome (*e.g.*, phosphatidylcholine/cholesterol) can disrupt the endosome to release its contents into the cytoplasm.

One approach to this problem has involved the use of cationic lipid vesicles. These cationic lipid vesicles form "complexes" with DNA, including plasmids and oligonucleotides. These complexes are not liposomes (*i.e.* an intact bilayer encapsulating an aqueous space) but are aggregates of lipid and DNA held together by electrostatic attraction between the cationic lipid and anionic nucleic acid. Recent literature indicates that complexes of cationic lipids in association with a fusogenic factor such as phosphatidylethanolamine or a fusion protein is required to achieve a significant antisense effect or gene transfection. Farhood, *et al.*, *Biochim. Biophys. Acta.* 1235:289-295 (1995); Felgner, *et al.*, *J. Biol. Chem.* 269:2550-2561 (1994); and Wrobel, *et al.*, *Biochim. Biophys. Acta.* 1235:296-304 (1995).

Still other studies involving intravenous administration of antisense oligonucleotides in monkeys have indicated certain toxicities associated with this practice (see Galbraith, *et al.*, *Antisense Res. Dev.* 4:201-206 (1994) and Cornish, *et al.*, *Antisense Res. Dev.* 3:239-247 (1993)). The most likely cause for the observed toxicities is the activation of the complement system, releasing C3a and C5a, vasoactive cleavage products of C3 and C5, respectively. In this regard, both DNA and RNA have been shown to activate complement in various *in vitro* assays.

The complement system is a multi-protein cascade which serves as one of the first lines of defense against foreign particles which have entered the blood. The two basic mechanisms by which complement attacks foreign particles are by opsonization and cell lysis. Opsonization involves the covalent attachment of complement fragments, principally C3b and iC3b, to the surface of a particle or cell, which is then recognized by corresponding receptors present on macrophages. Cell lysis involves the assembly of a multiprotein complex, C5b-9, which perforates cell membranes and generates a pore.

Relatively little work has been published concerning the use of neutral phospholipid vesicles (liposomes) as delivery systems for antisense oligonucleotides (see, Juliano, *et al.*, *Antisense Research & Development* 2:165-176 (1992)). Oligonucleotides are usually 15-25 bases in length and highly charged which means they cannot cross membranes by passive diffusion. The target site for antisense drugs is either the cytoplasm or nucleus and consequently the plasma membrane acts as an effective barrier. Despite this, some free antisense oligonucleotides have been shown active *in vitro* and *in vivo* (see, Juliano, *et al.*, *Antisense Research & Development* 2:165-176 (1992); Bennett, *et al.*, *J. Immunol.* 152:3530-3540 (1994); Bennett, *et al.*, *Adv. Pharmacol.* 28:1-43

(1994); and Stepkowski, *et al.*, *J. Immunol.* 153:5336-5346 (1994)), furthermore there is some evidence that a receptor mediated uptake mechanism for oligonucleotides may be responsible. Even if a target cell or tissue does exhibit an ability to endocytose antisense molecules the process is not efficient and biological efficacy is normally only observed at very high concentrations. This is most likely because even after endocytosis the nucleotide must still cross the endosomal membrane to reach the cytoplasm before it is degraded by lysosomal enzymes which occurs at a very low frequency. In the case of anti ICAM-1 the free oligonucleotide does not appear to penetrate endothelial cells in culture but when complexed with cationic lipid/phosphatidylethanolamine (PE) complexes extensive uptake is observed. Such cationic lipid/PE complexes are known to disrupt intracellular endosomes and deliver nucleic acids into the cytoplasm. Once in the cytoplasm antisense oligonucleotides rapidly diffuse into the nucleus (Sixou, *et al.*, *Nuc. Acids Res.* 22:662-668 (1994)) and this is also observed for anti ICAM-1 delivered by aggregates or complexes of the oligonucleotides and lipids (see Bennett, *et al.*, *Mol. Pharmacol.* 41:1023-1033 (1992)). As noted above, these latter complexes or aggregates are not liposome formulations and do not use encapsulation methods for construction of the complex. Moreover, the complexes (which are not encapsulated oligonucleotide systems) have not been shown to disrupt endosomes and as a result, will only show intracellular delivery of the oligonucleotide in *in vitro* studies.

Extensive modifications to the structure of oligonucleotides have been made in an attempt to improve stability *in vivo* and to enhance membrane permeability. For example the phosphorothioates, noted above, in which an oxygen atom is replaced by a sulphur in the phosphate backbone, exhibit increased resistance to nucleases and are more stable *in vivo* than normal phosphodiester oligonucleotides (Juliano, *et al.*, *Antisense Research & Development* 2:165-176 (1992)). Another example are nucleic acid methylphosphonates, which are not only nuclease resistant but also hydrophobic analogues of phosphodiesters and therefore expected to be more membrane permeable (see, Hughes, *et al.*, *J. Pharm. Sci.* 83:597-600 (1994) and Tari, *et al.*, *Blood* 84:601-607 (1994)). Despite these developments, antisense oligonucleotide therapy is slow to develop mainly because it is difficult to get therapeutic drug levels into cells in target tissue and toxic side effects persist such as complement activation which restrict dosing.

Attractive targets for antisense therapy include the nucleic acids which encode intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1

(VCAM-1), and endothelial leukocyte adhesion molecule-1 (ELAM-1). ICAM-1, which is a 90-110 kDa membrane glycoprotein involved in the trafficking of leukocytes out of the vasculature and in antigen presentation to T cells (see Osborn, *Cell* 56:907-910 (1990) and Springer, *Nature (Lond.)* 346:425-443 (1990)). ICAM-1 is normally expressed at low levels on the surface of endothelial cells, keratinocytes, fibroblasts and leukocytes. Expression of ICAM-1 is inducible by a number of cytokines, including IL-1 β , tumor necrosis factor- α and interferon- γ . Increased expression of ICAM-1 has been demonstrated in a variety of human diseases and has been shown to correlate with leukocyte infiltration in the diseased tissue.

What is needed in the art are new compositions and methods for the delivery of antisense molecules directed toward inhibiting the expression of cellular adhesion molecules. Such compositions should increase the serum stability of the antisense molecules and reduce toxic side effects such as complement activation. Surprisingly, the present invention provides such compositions and methods.

SUMMARY OF THE INVENTION

The present invention provides pharmaceutical compositions for the treatment of pathologic conditions associated with the overexpression of cellular adhesion molecules, such as ICAM-1 in a host. These pharmaceutical composition comprise an effective amount of an ICAM-1 antisense molecule encapsulated in a lipid mixture which is typically a liposome or lipid particle. The lipid mixture will typically comprise at least two members selected from the group consisting of phospholipids, sterols and cationic lipids.

In one group of embodiments, the antisense molecule is either a phosphorothioate molecule or a methyl phosphonate molecule, from about 15 to 50 nucleic acids, and is complementary to a portion of the 3'-untranslated region of ICAM-1.

For those embodiments in which the lipid mixture is a liposome composition, the liposome will preferably comprise phosphatidylcholine and cholesterol, more preferably egg phosphatidylcholine and cholesterol. For those embodiments in

which the lipid mixture is present as lipid particles, the particles will preferably comprise phospholipids and cationic lipids.

Additionally, the present invention provides methods for the treatment of pathologic conditions associated with the overexpression of ICAM-1 in a host. Such conditions include Alzheimer's disease, multiple sclerosis, uveitis, Herpes keratitis, renal allograft rejection, glomerulonephritis, liver allograft rejection, viral hepatitis, alcoholic hepatitis, cholangitis, cardiac allograft rejection, atherosclerotic plaques, rheumatoid arthritis, Grave's disease, Hashimoto's thyroiditis, psoriasis, scleroderma, graft v host disease, contact dermatitis, lichen planus, fixed drug eruption, mycosis fungoides, and alopecia areata.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates a spin column elution profile of encapsulated antisense. 50 μ L of encapsulated antisense was applied to a 1 mL Biogel A15m, 200-400 mesh, spin column and separated. Lipid was determined by phosphate analysis (O), and oligonucleotide was detected by measuring A_{260} , after Bligh and Dyer extraction (●).

Figure 2 illustrates a purified liposomal antisense preparation. Liposome-encapsulated antisense was "purified" on DEAE-sepharose CL-6B columns. Removal of free antisense was assessed by size exclusion chromatography on 1 mL Biogel A15m column. Lipid was determined by phosphate analysis (O), and oligonucleotide was detected by measuring A_{260} , after Bligh and Dyer extraction (●).

Figure 3 shows a time course for leakage of encapsulated antisense. Leakage of encapsulated antisense was monitored at room temperature for 1 (O), 3 (■) and 5 (●) days.

Figure 4 shows the time course for leakage of encapsulated antisense at 4°C. Leakage of encapsulated antisense was monitored for 1 (O), 3 (□), and 5 (●) days, at 4°C.

Figure 5 illustrates complement activation by liposomes. Complement activation was investigated using an EPC/CH liposome preparation. Liposomes were 100 ± 30 nm. Lipid composition is expressed in molar ratios.

Figure 6 illustrates the ear swelling characteristics of ICR mice.

Measurements refer to increases in ear thickness with respect to baseline measurements (prior to ear challenge). Values are given for two separate experiments. Error bars represent the standard deviation of measurements from 4 mice.

Figure 7 illustrates the ear swelling characteristics of BALB/c mice.

Measurements refer to increases in ear thickness with respect to baseline measurements (prior to ear challenge). Error bars represent the standard deviation of measurements from 4 mice.

Figure 8 shows the liposome accumulation in the ears of ICR mice during

various time intervals of inflammation. Liposomes were injected into mice at 0 hr, 24 hr, and 48 hr after initiation of ear inflammation. Liposomes were allowed to circulate for 24 hr at which time ears were recovered, digested, and analyzed for radiolabeled lipid. Error bars represent the standard deviation of measurements from 4 mice.

Figure 9 shows the liposome accumulation in the ears of BALB/c mice

during various time intervals of inflammation. Liposomes were injected into mice at 0 hr, 24 hr, and 48 hr after initiation of ear inflammation. Liposomes were allowed to circulate for 24 hr at which time ears were recovered, digested, and analyzed for radiolabeled lipid. Error bars represent the standard deviation of measurements from 4 mice.

Figure 10 shows the MPO levels in the ears of ICR mice during

inflammation. At various times after the initiation of inflammation, inflamed ears (■) and control ears (●) were recovered, homogenized, and assayed for MPO activity. Error bars represent the standard deviation of measurements from 4 mice.

Figure 11 shows cell infiltration into the inflamed ear of ICR mice during

inflammation. Bone marrow cells and circulating leukocytes were labeled 24 hr prior to the onset of inflammation. At various times after the initiation of inflammation, ears were recovered, digested, and analyzed for radiolabeled cells by liquid scintillation counting. Error bars represent the standard deviation of measurements from 4 mice.

Figure 12 shows a typical inflammation experiment involving ICR mice.

The following parameters were measured: ear swelling (Δ); liposome accumulation in inflamed () and non-inflamed (■) ears; and cell infiltration (O).

Figure 13 shows liposome accumulation in the ears of ICR mice during

the first 24 hours of inflammation. DSPC:CH liposomes were injected into mice

immediately after initiation of ear inflammation. At various times mice were sacrificed and the ears were collected and analyzed (inflamed () and non-inflamed (■) ears). Error bars represent the standard deviation of measurements from 4 mice.

Figure 14 illustrates the circulation clearance rates of free [³H]-antisense and liposome encapsulated [³H]-antisense.

Figure 15 illustrates the tissue biodistribution of free [³H]-antisense (Isis 2302).

Figure 16 illustrates the tissue biodistribution profiles for both the lipid and antisense portions of a liposome encapsulated antisense formulation.

Figure 17 illustrates the ability of free antisense to inhibit ear inflammation.

Figure 18 illustrates the efficacy of free and encapsulated ICAM-1 antisense formulations in reducing ear inflammation in mice.

Figure 19 is a bar graph showing edema formation (based on ear weights) in mice treated with free and encapsulated antisense.

DETAILED DESCRIPTION OF THE INVENTION

Abbreviations and Definitions

Abbreviations used herein have the following meanings: i.v., intravenous; DC-Chol, 3 β -(N-(N',N'-dimethylaminoethane)carbamoyl)cholesterol (see, Gao, *et al.*, *Biochem. Biophys. Res. Comm.* 179:280-285 (1991)); DDAB, N,N-distearyl-N,N-dimethylammonium bromide; DMRIE, N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide; DODAC, N,N-dioleoyl-N,N-dimethylammonium chloride (see commonly owned patent application USSN 08/316,399, incorporated herein by reference); DOGS, dioctadecylamidoglycyl carboxyspermine; DOPE, 1,2-*sn*-dioleoylphosphatidylethanolamine; DOSPA, N-(1-(2,3-dioleoyloxy)propyl)-N-(2-(sperminecarboxamido)ethyl)-N,N-dimethylammonium trifluoroacetate; DOTAP, N-((2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride; DOTMA, N-((2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride; EPC, egg phosphatidylcholine; RT, room temperature; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HBS, HEPES buffered saline (150 mM NaCl and 20 mM

HEPES); PEG-Cer-C₂₀, 1-O-(2'-(ω -methoxypolyethyleneglycol)succinoyl)-2-N-arachidoyl-sphingosine; PEG-Cer-C₁₄, 1-O-(2'-(ω -methoxypolyethyleneglycol)succinoyl)-2-N-myristoyl-sphingosine; PBS, phosphate-buffered saline; EGTA, ethylenebis(oxyethylenenitrilo)-tetraacetic acid; OGP, n-octyl β -D-glycopyranoside
5 (Sigma Chemical Co., St. Louis, MO); POPC, palmitoyl oleoyl phosphatidylcholine (Northern Lipids, Vancouver, BC); QELS, quasielastic light scattering; TBE, 89 mM Tris-borate with 2 mM EDTA; and EDTA, Ethylenediaminetetraacetic acid (Fisher Scientific, Fair Lawn, NJ); RES, reticuloendothelial system; SPDP, N-succinimidyl 3-(2-pyridyldithio) propionic acid; DTT, dithiothreitol; NEM, N-ethylmaleimide; Chol,
10 cholesterol; DMPC, 1,2-*sn*-dimyristoylphosphatidylcholine; DSPC, 1,2-*sn*-distearoylphosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; MLV, multilammellar vesicles; PEG-DSPE, poly(ethylene glycol)-modified distearoylphosphatidylethanolamine; LUV, large unilamellar vesicles; SUV, small unilamellar vesicles; PEG, poly(ethylene glycol); ³H-CHE, ³H-Cholesteryl hexadecyl
15 ether.

The term "oligonucleotide" refers to a single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. It includes both self-replicating plasmids, infectious polymers of DNA or RNA and non-functional DNA or RNA. When used to refer to oligonucleotides, the terms
20 "phosphorothioate" and "methyl phosphonate" refer to those oligonucleotides in which a phosphodiester internucleotide linkage has been modified by replacing at least one of the non-bridged oxygens of the internucleotide linkage with sulfur or a methyl group, respectively. Preferably at least 10% of the internucleotide linkages are modified, more preferably at least 30% of the linkages are so modified. Most preferably, at least 50%
25 of the linkages are modified.

The term "complementary" means that one nucleic acid hybridizes selectively to another nucleic acid. Selectivity of hybridization exists when hybridization (or base pairing) occurs that is more selective than total lack of specificity. Typically, selective hybridization will occur when there is at least about 55% paired bases over a
30 stretch of at least 14-25 nucleotides, preferably at least about 65%, more preferably at least about 75%, and most preferably at least about 90%. See, M. Kanehisa *Nucleic Acids Res.* 12:203 (1984), incorporated herein by reference.

The term "lipid" refers to any fatty acid derivative which is capable of forming a bilayer such that a hydrophobic portion of the lipid material orients toward the bilayer while a hydrophilic portion orients toward the aqueous phase. Hydrophilic characteristics derive from the presence of phosphato, carboxylic, sulfato, amino, sulfhydryl, nitro, and other like groups. Hydrophobicity could be conferred by the inclusion of groups that include, but are not limited to, long chain saturated and unsaturated aliphatic hydrocarbon groups and such groups substituted by one or more aromatic, cycloaliphatic or heterocyclic group(s). Preferred lipids are phosphoglycerides and sphingolipids, representative examples of which include phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, palmitoyloleoyl phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, distearoylphosphatidylcholine or dilinoleoylphosphatidylcholine could be used. Other compounds lacking in phosphorus, such as sphingolipid and glycosphingolipid families are also within the group designated as lipid. Additionally, the amphipathic lipids described above may be mixed with other lipids including triglycerides and sterols.

The term "cationic lipid" refers to any of a number of lipid species which carry a net positive charge at physiological pH. Such lipids include, but are not limited to, DODAC, DOTMA, DDAB, DOTAP, DC-Chol and DMRIE. Additionally, a number of commercial preparations of cationic lipids are available which can be used in the present invention. These include, for example, LIPOFECTIN® (commercially available cationic liposomes comprising DOTMA and DOPE, from GIBCO/BRL, Grand Island, New York, USA); LIPOFECTAMINE® (commercially available cationic liposomes comprising DOSPA and DOPE, from GIBCO/BRL); and TRANSFECTAM® (commercially available cationic liposomes comprising DOGS from Promega Corp., Madison, Wisconsin, USA).

As used herein, the phrase "pathologic conditions associated with the overexpression of ICAM-1", is meant to include diseases of the central nervous system (e.g. Alzheimer's disease and multiple sclerosis), the eye (e.g. uveitis and Herpes keratitis), the kidney (e.g. renal allograft rejection and glomerulonephritis), the liver (e.g. liver allograft rejection, viral hepatitis, alcoholic hepatitis, cholangitis), the heart (e.g. cardiac allograft rejection and atherosclerotic plaques), the bone (e.g. rheumatoid arthritis), the thyroid (e.g. Grave's disease and Hashimoto's thyroiditis), and the skin

(e.g. psoriasis, scleroderma, graft v host disease, contact dermatitis, lichen planus, fixed drug eruption, mycosis fungoides, and alopecia areata).

As used herein, the term "host" refers to a human, rat, mouse, dog, cow, sheep, horse, cat and goat.

5 Description of the Embodiments

The present invention derives from the surprising discovery that antisense molecules which are encapsulated in a liposome or lipid particle composition can be delivered to a site of inflammation in response to overexpression of ICAM-1 and thereby reduce the associated inflammation. It was particularly surprising that liposome
10 formulations which consist essentially of charge neutral lipids and a sterol (e.g., cholesterol) would be effective for antisense delivery in view of the conventional wisdom that cationic liposome formulations or formulations having fusogenic lipids or proteins are necessary for cell or endosome fusion.

Accordingly, the present invention provides pharmaceutical compositions
15 for the treatment of conditions associated with the overexpression of cellular adhesion molecules, preferably ICAM-1. These compositions comprise an antisense oligonucleotide encapsulated in a lipid mixture. The lipid mixture can be in either of two forms. The first is a conventional liposome, which is preferably charge neutral, consists essentially of neutral phospholipids and a sterol (e.g., cholesterol) and which can be
20 passively loaded with an antisense molecule. The second form is a lipid particle which comprises phospholipids, cationic lipids, sterols and combinations thereof.

Recently, others have described a cationic liposomal preparations of N-((2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA) for the delivery of an ICAM-1 antisense molecule (see, Bennett, *et al.*, *Mol. Pharmacol.* 41:1023-1033
25 (1992)). The preparation used by Bennett, *et al.*, was a 1:1 mixture of DOTMA and dioleoyl phosphatidylethanolamine which formed 250 nm liposomes. These systems provided complexes of the antisense molecules which were not encapsulated. Moreover, these systems are useful only *in vitro* for intracellular delivery as they do not disrupt endosomes *in vivo*.

ANTISENSE OLIGONUCLEOTIDES

The antisense oligonucleotides which are useful in the present invention are those oligonucleotides which are complementary to a portion of a mammalian nucleic acid encoding cellular adhesion molecules such as ELAM-1 (human), VCAM-1 (human) and ICAM-1 (human and mouse) which are provided as Sequence I.Ds. No. 1, 3, 5 and 7, respectively).

ELAM-1 is a 115-kDa membrane glycoprotein which is a member of the selecting family of membrane glycoproteins (see, Bevilacqua, *et al.*, *Science*, 243:1160-1165 (1989)). The amino terminal region of ELAM-1 contains sequences with homologies to members of lectin-like proteins, followed by a domain similar to epidermal growth factor, followed by six tandem 60-amino acid repeats similar to those found in complement receptors 1 and 2. ELAM-1 is encoded by a 3.9-kb mRNA. The 3'-untranslated region of ELAM-1 mRNA contains several sequence motifs ATTTA which are responsible for the rapid turnover of cellular mRNA consistent with the transient nature of ELAM-1 expression.

ELAM-1 exhibits a limited cellular distribution and has only been identified on vascular endothelial cells. Like ICAM-1, ELAM-1 is inducible by a number of cytokines including tumor necrosis factor, interleukin-1 and lymphotoxin and bacterial lipopolysaccharide. Unlike ICAM-1, ELAM-1 is not induced by gamma-interferon. The kinetics of ELAM-1 mRNA induction and disappearance in human umbilical endothelial cells precedes the appearance and disappearance of ELAM-1 on the cell surface.

VCAM-1 is a 110-kDa membrane glycoprotein encoded by a 3.2-kb mRNA. It appears to be encoded by a single-copy gene which can undergo alternative splicing to yield products with either six or seven immunoglobulin domains (see Osborn, *et al.*, *Cell* 59:1203-1211 (1989)). The receptor for VCAM-1 is proposed to be CD29 (VLA-4) as demonstrated by monoclonal antibodies which bind to CD29 and block the adherence of Ramos cells to VCAM-1. VCAM-1 is expressed primarily on vascular endothelial cells and is also regulated by treatment with cytokines (see, Rice, *et al.*, *Science* 246:1303-1306 (1989) and Rice, *et al.*, *J. Exp. Med.* 171:1369-1374 (1990)). Increased expression appears to be due to induction of the mRNA.

Human ICAM-1 is encoded by a 3.3-kb mRNA resulting in the synthesis of a 55,219 dalton protein. ICAM-1 is heavily glycosylated through N-linked

glycosylation sites. The mature protein has an apparent molecular mass of 90 kDa as determined by gel electrophoresis (see Staunton, *et al.*, *Cell* 52:925-933 (1988)).

ICAM-1 is a member of the immunoglobulin supergene family, containing 5 immunoglobulin-like domains at the amino terminus, followed by a transmembrane domain and a cytoplasmic domain. The primary binding site for LFA-1 and rhinovirus are found in the first immunoglobulin-like domain. However, the binding sites appear to be distinct.

ICAM-1 exhibits a broad tissue and cell distribution, and may be found on white blood cells, endothelial cells, fibroblast, keratinocytes and other epithelial cells. The expression of ICAM-1 can be regulated on vascular endothelial cells, fibroblasts, keratinocytes, astrocytes and several cell lines by treatment with bacterial lipopolysaccharide and cytokines such as interleukin-1, tumor necrosis factor, gamma-interferon, and lymphotoxin (see, *e.g.*, Frohman, *et al.*, *J. Neuroimmunol.* 23:117-124 (1989)).

In preferred embodiments, the antisense oligonucleotide is complementary to the 3'-untranslated region of human ICAM-1 mRNA and contains from about 10 to about 50 nucleotides, more preferably about 15 to about 30 nucleotides. In particularly preferred embodiments, the antisense oligonucleotide is a phosphorothioate oligonucleotide or a methyl phosphonate oligonucleotide. Phosphorothioate oligonucleotides (PS-oligos) are those oligonucleotides in which one of the non-bridged oxygens of the internucleotide linkage has been replaced with sulfur. These PS-oligos are resistant to nuclease degradation, yet retain sequence-specific activity. Similarly, methyl phosphonate oligonucleotides (MeP-oligos) are those oligonucleotides in which one of the non-bridged oxygens of the internucleotide linkage has been replaced by a methyl group. These MeP-oligos have also proven to be more nuclease resistant than their natural phosphodiester linked derivatives. A number of antisense oligonucleotides which are directed toward inhibiting the production of ICAM-1, as well as VCAM-1 and ELAM-1, and which are useful in the present invention have been described in PCT applications: PCT/US91/05209; PCT/US94/09026; PCT/US94/12797 and PCT/US93/08101, the disclosures of each being incorporated herein by reference.

The antisense oligonucleotides used in the present invention may be synthesized in solid phase or in solution. Generally, solid phase synthesis is preferred. Detailed descriptions of the procedures for solid phase synthesis of oligonucleotides by

phosphite-triester, phosphotriester, and H-phosphonate chemistries are widely available. See, for example, Itakura, U.S. Pat. No. 4,401,796; Caruthers, *et al.*, U.S. Pat. Nos. 4,458,066 and 4,500,707; Beaucage, *et al.*, *Tetrahedron Lett.*, 22:1859-1862 (1981); Matteucci, *et al.*, *J. Am. Chem. Soc.*, 103:3185-3191 (1981); Caruthers, *et al.*, *Genetic Engineering*, 4:1-17 (1982); Jones, chapter 2, Atkinson, *et al.*, chapter 3, and Sproat, *et al.*, chapter 4, in *Oligonucleotide Synthesis: A Practical Approach*, Gait (ed.), IRL Press, Washington D.C. (1984); Froehler, *et al.*, *Tetrahedron Lett.*, 27:469-472 (1986); Froehler, *et al.*, *Nucleic Acids Res.*, 14:5399-5407 (1986); Sinha, *et al.*, *Tetrahedron Lett.*, 24:5843-5846 (1983); and Sinha, *et al.*, *Nucl. Acids Res.*, 12:4539-4557 (1984) which are incorporated herein by reference.

Generally, the timing of delivery and concentration of monomeric nucleotides utilized in a coupling cycle will not differ from the protocols typical for commercial phosphoramidites used in commercial DNA synthesizers. In these cases, one may merely add the solution containing the monomers to a receptacle on a port provided for an extra phosphoramidite on a commercial synthesizer (*e.g.*, model 380B, Applied Biosystems, Foster City, California, U.S.A.). However, where the coupling efficiency of a particular monomer is substantially lower than the other phosphoramidites, it may be necessary to alter the timing of delivery or the concentration of the reagent in order to optimize the synthesis. Means of optimizing oligonucleotide synthesis protocols to correct for low coupling efficiencies are well known to those of skill in the art. Generally one merely increases the concentration of the reagent or the amount of the reagent delivered to achieve a higher coupling efficiency. Methods of determining coupling efficiency are also well known. For example, where the 5'-hydroxyl protecting group is dimethoxytrityl (DMT), coupling efficiency may be determined by measuring the DMT cation concentration during the acidic removal of the DMT group. DMT cation concentration is usually determined by spectrophotometrically monitoring the acid wash. The acid/DMT solution is a bright orange color. Alternatively, since capping prevents further extension of an oligonucleotide where coupling has failed, coupling efficiency may be estimated by comparing the ratio of truncated to full length oligonucleotides utilizing, for example, capillary electrophoresis or HPLC.

Solid phase oligonucleotide synthesis may be performed using a number of solid supports. A suitable support is one which provides a functional group for the attachment of a protected monomer which will become the 3' terminal base in the

synthesized oligonucleotide. The support must be inert to the reagents utilized in the particular synthesis chemistry. Suitable supports are well known to those of skill in the art. Solid support materials include, but are not limited to polyacryloylmorpholide, silica, controlled pore glass (CPG), polystyrene, polystyrene/latex, and carboxyl modified teflon. Preferred supports are amino-functionalized controlled pore glass and carboxyl-functionalized teflon.

Solid phase oligonucleotide synthesis requires, as a starting point, a fully protected monomer (*e.g.*, a protected nucleoside) coupled to the solid support. This coupling is typically through the 3'-hydroxyl. Typically, a linker group is covalently bound to the 3'-hydroxyl on one end and covalently bound to the solid support on the other end. The first synthesis cycle then couples a nucleotide monomer, via its 3'-phosphate, to the 5'-hydroxyl of the bound nucleoside through a condensation reaction that forms a 3'-5' phosphodiester linkage. Subsequent synthesis cycles add nucleotide monomers to the 5'-hydroxyl of the last bound nucleotide. In this manner an oligonucleotide is synthesized in a 3' to 5' direction producing a "growing" oligonucleotide with its 3' terminus attached to the solid support.

Numerous means of linking nucleoside monomers to a solid support are known to those of skill in the art, although monomers covalently linked through a succinate or hemisuccinate to controlled pore glass are generally preferred. Conventional protected nucleosides coupled through a hemisuccinate to controlled pore glass are commercially available from a number of sources (*e.g.*, Glen Research, Sterling, Vermont, U.S.A.; Applied Biosystems, Foster City, California, U.S.A.; and Pharmacia LKB, Piscataway, New Jersey, U.S.A.).

Once the full length oligonucleotide is synthesized, the oligonucleotide is deprotected and cleaved from the solid support prior to use. Cleavage and deprotection may occur simultaneously or sequentially in any order. The two procedures may be interspersed so that some protecting groups are removed from the oligonucleotide before it is cleaved off the solid support and other groups are deprotected from the cleaved oligonucleotide in solution. The sequence of events depends on the particular blocking groups present, the particular linkage to a solid support, and the preferences of the individuals performing the synthesis. Where deprotection precedes cleavage, the protecting groups may be washed away from the oligonucleotide which remains bound on the solid support. Conversely, where deprotection follows cleavage, the removed

protecting groups will remain in solution with the oligonucleotide. Often the oligonucleotide will require isolation from these protecting groups prior to use.

In a preferred embodiment, and most commercial DNA syntheses, the protecting group on the 5'-hydroxyl is removed at the last stage of synthesis. The oligonucleotide is then cleaved off the solid support, and the remaining deprotection occurs in solution. Removal of the 5'-hydroxyl protecting group typically requires treatment with the same reagent utilized throughout the synthesis to remove the terminal 5'-hydroxyl protecting groups prior to coupling the next nucleotide monomer. Where the 5'-hydroxyl protecting group is a dimethoxytrityl group, deprotection can be accomplished by treatment with acetic acid, dichloroacetic acid or trichloroacetic acid.

Where the oligonucleotide is a ribonucleotide and the 2'-hydroxyl group is blocked with a tert-butyldimethylsilyl (TBDMS) moiety, the latter group may be removed using tetrabutylammonium fluoride in tetrahydrofuran at the end of synthesis. See Wu, *et al.*, *J. Org. Chem.* 55:4717-4724 (1990). Phenoxyacetyl protecting groups can be removed with anhydrous ammonia in alcohol (under these conditions the TBDMS groups are stable and the oligonucleotide is not cleaved). The benzoyl protecting group of cytidine is also removed with anhydrous ammonia in alcohol.

Cleaved and fully deprotected oligonucleotides may be used directly (after lyophilization or evaporation to remove the deprotection reagent) or they may be purified prior to use. Purification of synthetic oligonucleotides is generally desired to isolate the full length oligonucleotide from the protecting groups that were removed in the deprotection step and, more importantly, from the truncated oligonucleotides that were formed when oligonucleotides that failed to couple with the next nucleotide monomer were capped during synthesis.

Oligonucleotide purification techniques are well known to those of skill in the art. Methods include, but are not limited to, thin layer chromatography (TLC) on silica plates, gel electrophoresis, size fractionation (*e.g.*, using a Sephadex column), reverse phase high performance liquid chromatography (HPLC) and anion exchange chromatography (*e.g.*, using the mono-Q column, Pharmacia-LKB, Piscataway, New Jersey, U.S.A.). For a discussion of oligonucleotide purification see McLaughlin, *et al.*, chapter 5, and Wu, *et al.*, chapter 6 in *Oligonucleotide Synthesis: A Practical Approach*, Gait (ed.), IRL Press, Washington, D.C., (1984).

To test the therapeutic ability of liposomes as delivery vehicles for antisense molecules to ICAM-1, ISIS Pharmaceuticals donated their antisense molecules ISIS 2302 (human, Sequence I.D. No. 9) and 3082 (murine, Sequence I.D. No. 10) towards the project. These oligonucleotides are phosphorothioate molecules in which each phosphodiester linkage is a phosphorothioate diester linkage. Additionally, each oligonucleotide consists of 20 nucleotides specifically designed to bind to a sequence in the 3' untranslated region of ICAM-1 mRNA. Currently it is theorized that this antisense molecule acts posttranscriptionally to inhibit the expression of ICAM-1 via 2 possible mechanisms: inhibiting the formation of a stabilizing stem-loop structure necessary for mRNA stability, and by increasing the degradation of the target mRNA via increased RNase-H degradation.

Preparation of pharmaceutical compositions containing the antisense oligonucleotides will typically involve either encapsulating the oligonucleotide in a liposome or forming a lipid particle in which the oligonucleotide is coated with a lipid mixture.

LIPOSOME-ENCAPSULATED ANTISENSE OLIGONUCLEOTIDES

The liposomes which are used in the present invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids, preferably neutral phospholipids, and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of, e.g., liposome size and stability of the liposomes in the bloodstream.

Typically, the major lipid component in the liposomes is phosphatidylcholine. Phosphatidylcholines having a variety of acyl chain groups of varying chain length and degree of saturation are available or may be isolated or synthesized by well-known techniques. In general, less saturated phosphatidylcholines are more easily sized, particularly when the liposomes must be sized below about 0.3 microns, for purposes of filter sterilization. Phosphatidylcholines containing saturated fatty acids with carbon chain lengths in the range of C_{14} to C_{22} are preferred. Phosphatidylcholines with mono or diunsaturated fatty acids and mixtures of saturated and unsaturated fatty acids may also be used. Other suitable lipids include phosphonolipids in which the fatty acids are linked to glycerol via ether linkages rather than ester linkages. Liposomes useful in the present invention may also be composed of

sphingomyelin or phospholipids with head groups other than choline, such as ethanolamine, serine, glycerol and inositol. Preferred liposomes will include a sterol, preferably cholesterol, at molar ratios of from 0.1 to 1.0 (cholesterol:phospholipid). Most preferred liposome compositions are egg phosphatidylcholine/cholesterol, distearoylphosphatidylcholine/cholesterol, dipalmitoylphosphatidylcholine/cholesterol, and sphingomyelin/cholesterol. Methods used in sizing and filter-sterilizing liposomes are discussed below.

The liposomes can be prepared by any of the techniques now known or subsequently developed for preparing liposomes. For example, the liposomes can be formed by the conventional technique for preparing multilamellar lipid vesicles (MLVs), that is, by depositing one or more selected lipids on the inside walls of a suitable vessel by dissolving the lipids in chloroform and then evaporating the chloroform, and by then adding the aqueous solution which is to be encapsulated to the vessel, allowing the aqueous solution to hydrate the lipid, and swirling or vortexing the resulting lipid suspension. This process engenders a mixture including the desired liposomes.

Alternatively, techniques used for producing large unilamellar lipid vesicles (LUVs), such as reverse-phase evaporation, infusion procedures, and detergent dilution, can be used to produce the liposomes. A review of these and other methods for producing lipid vesicles can be found in the text *Liposome Technology*, Volume I, Gregory Gregoriadis Ed., CRC Press, Boca Raton, Florida, (1984), which is incorporated herein by reference. For example, the lipid-containing particles can be in the form of steroidal lipid vesicles, stable plurilamellar lipid vesicles (SPLVs), monophasic vesicles (MPVs), or lipid matrix carriers (LMCs) of the types disclosed in Lenk, *et al.* U.S. Patent No. 4,522,803, and Fountain, *et al.* U.S. Patent Nos. 4,588,578 and 4,610,868, the disclosures of which are incorporated herein by reference. A particularly preferred method for preparing LUVs is described in U.S. Patent No. 5,008,050.

Additionally, in the case of MLVs, if desired, the liposomes can be subjected to multiple (five or more) freeze-thaw cycles to enhance their trapped volumes and trapping efficiencies and to provide a more uniform interlamellar distribution of solute (Mayer, *et al.*, *J. Biol. Chem.* 260:802-808 (1985)).

Following liposome preparation, the liposomes may be sized to achieve a desired size range and relatively narrow distribution of liposome sizes. In preferred

embodiments, the liposomes will have diameters of from about 50 to about 150 nm, more preferably from about 75 to about 125 nm.

Several techniques are available for sizing liposomes to a desired size. One sizing method is described in U.S. Pat. No. 4,737,323, incorporated herein by reference. Sonicating a liposome suspension either by bath or probe sonication produces a progressive size reduction down to small unilamellar vesicles (SUVs) less than about 0.05 microns in size. Homogenization is another method which relies on shearing energy to fragment large liposomes into smaller ones. In a typical homogenization procedure, multilamellar vesicles are recirculated through a standard emulsion homogenizer until selected liposome sizes, typically between about 0.1 and 0.5 microns, are observed. In both methods, the particle size distribution can be monitored by conventional laser-beam particle size determination.

Extrusion of liposome through a small-pore polycarbonate membrane or an asymmetric ceramic membrane is also an effective method for reducing liposome sizes to a relatively well-defined size distribution (see, U.S. Patent No. 5,008,050 and Hope, *et al.*, in: *Liposome Technology*, vol. 1, 2d ed. (G. Gregoriadis, Ed.) CRC Press, pp. 123-139 (1992), the disclosures of which are incorporated herein by reference). Typically, the suspension is cycled through the membrane one or more times until the desired liposome size distribution is achieved. The liposomes may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in liposome size. For use in the present inventions, liposomes having a size of from about 0.05 microns to about 0.15 microns are preferred. Other useful sizing methods such as sonication, solvent vaporization or reverse phase evaporation are known to those of skill in the art.

Liposomes prepared for use in the methods and pharmaceutical compositions of the present invention may be dehydrated for longer storage. The liposomes are preferably dehydrated under reduced pressure using standard freeze-drying equipment or equivalent apparatus. The lipid vesicles and their surrounding medium can also be frozen in liquid nitrogen before being dehydrated or not, and placed under reduced pressure. Dehydration without prior freezing takes longer than dehydration with prior freezing, but the overall process is gentler without the freezing step, and thus there is subsequently less damage to the lipid vesicles and a smaller loss of the internal contents. Dehydration without prior freezing at room temperature and at a reduced pressure provided by a vacuum pump capable of producing a pressure of about 1 mm Hg

typically takes between approximately 24 and 36 hours, while dehydration with prior freezing under the same conditions generally takes between approximately 12 and 24 hours.

To ensure that the liposomes will survive the dehydration process without losing a substantial portion of their internal contents, it is important that one or more protective sugars be available to interact with the lipid vesicle membranes and keep them intact as the water in the system is removed. A variety of sugars can be used, including such sugars as trehalose, maltose, sucrose, glucose, lactose, and dextran. In general, disaccharide sugars have been found to work better than monosaccharide sugars, with the disaccharide sugars trehalose and sucrose being most effective. Other more complicated sugars can also be used. For example, aminoglycosides, including streptomycin and dihydrostreptomycin, have been found to protect lipid vesicles during dehydration.

Typically, one or more sugars are included as part of either the internal or external media of the lipid vesicles. Most preferably, the sugars are included in both the internal and external media so that they can interact with both the inside and outside surfaces of the liposomes' membranes. Inclusion in the internal medium is accomplished by adding the sugar or sugars to the buffer which becomes encapsulated in the lipid vesicles during the lipid vesicle formation process. Since in most cases this buffer also forms the bathing medium for the finished lipid vesicles, inclusion of the sugars in the buffer also makes them part of the external medium.

The amount of sugar to be used depends on the type of sugar used and the characteristics of the lipid vesicles to be protected. See, U.S. Patent No. 4,880,635 and Harrigan, *et al.*, *Chem. Phys. Lipids* 52:139-149 (1990), the disclosures of which are incorporated herein by reference. Persons skilled in the art can readily test various sugar types and concentrations to determine which combination works best for a particular lipid vesicle preparation. In general, sugar concentrations on the order of 100 mM and above have been found necessary to achieve the highest levels of protection. In terms of moles of membrane phospholipid, millimolar levels on the order of 100 mM correspond to approximately 5 moles of sugar per mole of phospholipid.

In the case of dehydration without prior freezing, if the lipid vesicles being dehydrated are of the type which have multiple lipid layers and if the dehydration is carried to an end point where between about 2% and about 5% of the original water in

the preparation is left in the preparation, the use of one or more protective sugars may be omitted.

Once the lipid vesicles have been dehydrated, they can be stored for extended periods of time until they are to be used. The appropriate temperature for storage will depend on the make up of the lipid vesicles and the temperature sensitivity of whatever materials have been encapsulated in the lipid vesicles. For example, as is known in the art, various oligonucleotides are heat labile, and thus dehydrated lipid vesicles containing such oligonucleotides should be stored under refrigerated conditions so that the potency of the agent is not lost. Also, for such agents, the dehydration process is preferably carried out at reduced temperatures, rather than at room temperature.

Methods of loading antisense oligonucleotides into liposomes will typically be carried out using an encapsulation technique in which the antisense oligonucleotide is placed into a buffer and added to a dried film of only lipid components. In this manner, the oligonucleotide will become encapsulated in the aqueous interior of the liposome. The buffer which is used in the formation of the liposomes can be any biologically compatible buffer solution of, for example, isotonic saline, phosphate buffered saline, or other low ionic strength buffers. Generally, the antisense oligonucleotide will be present in an amount of from about 0.01 ng/mL to about 200 mg/mL. The resulting liposomes with the antisense oligonucleotide incorporated in the aqueous interior or in the membrane are then optionally sized as described above.

Alternatively, the antisense oligonucleotide can be formulated in lipid particles such as those described in co-pending U.S. Ser. Nos. 08/484,282 and 08/485,458 each being filed on June 7, 1995 and incorporated herein by reference.

ANTISENSE LIPID PARTICLES

Antisense lipid particles can be prepared by combining an antisense oligonucleotide with cationic lipids in a detergent solution to provide a coated antisense-lipid complex. The complex is then contacted with phospholipids to provide a solution of detergent, an antisense-lipid complex and phospholipids, and the detergent is then removed to provide a solution of serum-stable antisense-lipid particles, in which the

antisense oligonucleotide is encapsulated in a lipid bilayer. The particles, thus formed, have a size of about 50-150 nm.

Alternatively, serum-stable antisense-lipid particles can be formed by preparing a mixture of cationic lipids and phospholipids in an organic solvent; contacting an aqueous solution of the antisense oligonucleotide with the mixture of cationic and phospholipids to provide a clear single phase; and removing the organic solvent to provide a suspension of antisense-lipid particles, in which the antisense oligonucleotide is encapsulated in a lipid bilayer, and the particles are stable in serum and have a size of about 50-150 nm.

In this aspect of the invention, cationic lipids which are useful will include, for example, DODAC, DOTMA, DDAB, DOTAP, DC-Chol, DORI and DMRIE. These lipids and related analogs, which are also useful in the present invention, have been described in co-pending USSN 08/316,399; U.S. Patent Nos. 5,208,036, 5,264,618, 5,279,833, 5,283,185, and 5,334,761, the disclosures of which are incorporated herein by reference. Additionally, a number of commercial preparations of cationic lipids are available and can be used in the present invention. These include, for example, LIPOFECTIN® (commercially available cationic liposomes comprising DOTMA and DOPE, from GIBCO/BRL, Grand Island, New York, USA); LIPOFECTAMINE® (commercially available cationic liposomes comprising DOSPA and DOPE, from GIBCO/BRL); and TRANSFECTAM® (commercially available cationic liposomes comprising DOGS from Promega Corp., Madison, Wisconsin, USA).

An initial solution of coated antisense-lipid complexes is formed by combining the antisense oligonucleotides with the cationic lipids in a detergent solution. The detergent solution is preferably an aqueous solution of a neutral detergent having a critical micelle concentration of 15-300 mM, more preferably 20-50 mM. Examples of suitable detergents include, for example, N,N'-((octanoylimino)-bis-(trimethylene))-bis-(D-gluconamide) (BIGCHAP); BRIJ 35; Deoxy-BIGCHAP; dodecylpoly(ethylene glycol) ether; Tween 20; Tween 40; Tween 60; Tween 80; Tween 85; Mega 8; Mega 9; Zwittergent® 3-08; Zwittergent® 3-10; Triton X-405; hexyl-, heptyl-, octyl- and nonyl- β -D-glucopyranoside; and heptylthioglucopyranoside; with octyl β -D-glucopyranoside being the most preferred. The concentration of detergent in the detergent solution is typically about 100 mM to about 2 M, preferably from about 200 mM to about 1.5 M.

The cationic lipids and antisense oligonucleotides will typically be combined to produce a charge ratio (+/-) of about 1:1 to about 20:1, preferably in a ratio of about 1:1 to about 12:1, and more preferably in a ratio of about 2:1 to about 6:1. Additionally, the overall concentration of antisense oligos in solution will typically be from about 25 $\mu\text{g/mL}$ to about 150 mg/mL , preferably from about 100 $\mu\text{g/mL}$ to about 50 mg/mL , and more preferably from about 100 $\mu\text{g/mL}$ to about 5 mg/mL . The combination of antisense oligos and cationic lipids in detergent solution is kept, typically at room temperature, for a period of time which is sufficient for the coated complexes to form. Alternatively, the antisense oligos and cationic lipids can be combined in the detergent solution and warmed to temperatures of up to about 37°C. For antisense oligos which are particularly sensitive to temperature, the coated complexes can be formed at lower temperatures, typically down to about 4°C.

The detergent solution of the coated antisense-lipid complexes is then contacted with non-cationic lipids to provide a detergent solution of antisense-lipid complexes and non-cationic lipids. The non-cationic lipids which are useful in this step include, diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, cephalin, cardiolipin, and cerebroside. In preferred embodiments, the phospholipids are diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide or sphingomyelin. The acyl groups in these lipids are preferably acyl groups derived from fatty acids having $\text{C}_{10}\text{-C}_{24}$ carbon chains. More preferably the acyl groups are lauroyl, myristoyl, palmitoyl, stearoyl or oleoyl. In particularly preferred embodiments, the non-cationic lipid will be 1,2-dioleoylphosphatidylethanolamine (DOPE), palmitoyl oleoyl phosphatidylcholine (POPC) or egg phosphatidylcholine (EPC). In the most preferred embodiments, the antisense-lipid particles will be fusogenic particles with enhanced properties *in vivo* and the phospholipid will be DOPE. In other preferred embodiments, the non-cationic lipids will further comprise polyethylene glycol-based polymers such as PEG 2000, PEG 5000 and polyethylene glycol conjugated to ceramides, as described in co-pending USSN 08/316,429, incorporated herein by reference...

The amount of phospholipid which is used in the present methods is typically about 2 to about 150 mg/mL of total lipids to about 2 to about 150 mg/mL of antisense oligonucleotide. Preferably the amount of total lipid is from about 50 to about 100 mg/mL to 50 to 100 mg/mL antisense.

Following formation of the detergent solution of antisense-lipid complexes and non-cationic lipids, the detergent is removed, preferably by dialysis. The removal of the detergent results in the formation of a lipid-bilayer which surrounds the antisense oligo providing serum-stable antisense-lipid particles which have a size of from about 50 nm to about 150 nm. The particles thus formed do not aggregate and are optionally sized to achieve a uniform particle size.

The serum-stable antisense-lipid particles can be sized by any of the methods available for sizing liposomes. The sizing may be conducted in order to achieve a desired size range and relatively narrow distribution of particle sizes.

Several techniques are available for sizing the particles to a desired size. One sizing method, used for liposomes and equally applicable to the present particles is described in U.S. Pat. No. 4,737,323, incorporated herein by reference. Sonicating a particle suspension either by bath or probe sonication produces a progressive size reduction down to particles of less than about 50 nm in size. Homogenization is another method which relies on shearing energy to fragment larger particles into smaller ones. In a typical homogenization procedure, particles are recirculated through a standard emulsion homogenizer until selected particle sizes, typically between about 60 and 80 nm, are observed. In both methods, the particle size distribution can be monitored by conventional laser-beam particle size discrimination, or QELS.

Extrusion of the particles through a small-pore polycarbonate membrane or an asymmetric ceramic membrane is also an effective method for reducing particle sizes to a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired particle size distribution is achieved. The particles may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in size.

As noted above, the serum-stable antisense-lipid particles can also be prepared by combining a mixture of cationic lipids and non-cationic lipids in an organic solvent; contacting an aqueous solution of the antisense oligonucleotide with the mixture of lipids to provide a clear single phase; and removing the organic solvent to provide a suspension of antisense-lipid particles, wherein the antisense oligonucleotide is encapsulated in a lipid bilayer, and the particles are stable in serum and have a size of from about 50 to about 150 nm.

The antisense oligos, cationic lipids and phospholipids which are useful in this group of embodiments are as described for the detergent dialysis preparative methods described above.

The selection of an organic solvent will typically involve consideration of solvent polarity and the ease with which the solvent can be removed at the later stages of particle formation. The organic solvent, which is also used as a solubilizing agent, is in an amount sufficient to provide a clear single phase mixture of antisense oligonucleotide and lipids. Suitable solvents include chloroform, dichloromethane, diethylether, cyclohexane, cyclopentane, benzene, toluene, methanol, or other aliphatic alcohols such as propanol, isopropanol, butanol, tert-butanol, iso-butanol, pentanol and hexanol. Combinations of two or more solvents may also be used in the present invention.

Contacting the antisense oligonucleotide with the organic solution of cationic and phospholipids is accomplished by mixing together a first solution of antisense oligonucleotide, which is typically an aqueous solution and a second organic solution of the lipids. One of skill in the art will understand that this mixing can take place by any number of methods, for example by mechanical means such as by using vortex mixers.

After the antisense oligonucleotide has been contacted with the organic solution of lipids, the organic solvent is removed, thus forming an aqueous suspension of serum-stable antisense-lipid particles. The methods used to remove the organic solvent will typically involve evaporation at reduced pressures or blowing a stream of inert gas (*e.g.*, nitrogen or argon) across the mixture.

The serum-stable antisense-lipid particles thus formed will typically be sized from about 50 nm to 150 nm. To achieve further size reduction or homogeneity of size in the particles, sizing can be conducted as described above.

ADMINISTRATION OF THE ANTISENSE FORMULATIONS

The present invention further provides methods for the treatment of pathologic conditions associated with the overexpression of ICAM-1 in a host. In these methods a pharmaceutical composition as described above is administered to the host. In preferred embodiments, the host is a mammal, more preferably a mouse, rat, human, horse, dog, cat, cow or pig. Still more preferably, the host is human. Preferred

antisense oligonucleotides, lipid mixtures and lipids are as described above for the compounds of the present invention.

5 The antisense oligonucleotide liposomes and lipid particles described above can be administered in any suitable manner, preferably with pharmaceutically acceptable carriers. One skilled in the art will appreciate that suitable methods of administering such compositions in the context of the present invention to an animal are available, and, although more than one route can be used to administer a particular composition, a particular route can provide a more immediate and more effective reaction than another route. Pharmaceutically acceptable carriers are also well-known to those who are skilled
10 in the art. The choice of carrier will be determined in part by the particular composition, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of the pharmaceutical composition of the present invention.

Formulations suitable for oral administration can consist of (a) liquid
15 solutions, such as an effective amount of the antisense oligonucleotide (in a liposome or lipid particle) dissolved in diluents, such as water, saline or PEG 400; (b) suspensions in an appropriate liquid; and (c) suitable emulsions.

Formulations suitable for parenteral administration, such as, for example, by intravenous, intramuscular, intradermal, intraperitoneal, and subcutaneous routes,
20 include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The formulations can be presented in unit-dose or multi-dose sealed
25 containers, such as ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water, for injections, immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described.

30 The dose administered to an animal, particularly a human, in the context of the present invention should be sufficient to effect a therapeutic response in the animal over a reasonable time frame. The dose will be determined by the strength of the particular compound employed and the condition of the animal, as well as the body

weight or surface area of the animal to be treated. The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects that might accompany the administration of a particular compound in a particular animal.

As noted, the antisense oligonucleotides (in liposome or lipid particle formulations) can be administered, for example, by intravenous infusion, orally, topically, intraperitoneally, intravesically or intrathecally.

For oral administration, the compositions of the present invention can be administered at the rate up to 3000 mg/m² body surface area, which approximates 6 grams/day in the average patient. A preferred rate is from 1 to 300 mg/m² body surface area. This can be accomplished via single or divided doses. For intravenous administration, such compounds can be administered at the rate of up to about 2500 mg/m²/d, preferably from about 0.1 to about 200 mg/m²/d. For intravesicle administration, such compounds can be administered at the rate of up to about 2500 mg/m²/d, preferably from about 0.1 to about 200 mg/m²/d. For topical administration, the rate can be up to about 2500 mg/m²/d, preferably from about 0.1 to about 200 mg/m²/d. The dose for inhalation/aerosol administration can be up to about 2500 mg/m²/d, preferably from about 0.1 to about 200 mg/m²/d. Direct intraperitoneal administration can be performed using up to about 3000 mg/m²/d, preferably from about 0.1 to about 100 mg/m²/d. The dose for reservoir administration to the brain or spinal fluid can be up to about 2000 mg/m²/d, preferably from about 0.1 to about 100 mg/m²/d. For slow release intraperitoneal or subcutaneous administration, the dose can be from about 0.1 to about 5000 mg/day in a bolus, preferably from about 1.0 to about 200 mg/day. For intrathecal administration, the dose can be up to about 2000 mg/m²/d, preferably from about 0.1 to about 100 mg/m²/d.

The following examples are offered solely for the purposes of illustration, and are intended neither to limit nor to define the invention.

EXAMPLES

Materials

1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and egg phosphatidylcholine (EPC) was purchased from Avanti Polar Lipids (Alabaster, Alabama,

USA). Cholesterol, N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid) (HEPES), SEPHAROSE® CL-4B, SEPHADEX® G-25, SEPHADEX® G-50 and dithiothreitol (DTT) were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). ³H-Cholesteryl hexadecyl ether (³H-CHE) was obtained from Amersham (Oakville, Ontario, Canada). Female ICR and Balb/c mice were obtained from Harley-Sprague Davis.

Buffers

5X Veronal Buffered Saline (VBS). Mix 85.0 g of NaCl, 3.75 g of sodium barbitone, and 5.75 g of barbitone in 1.6 L of distilled water. Heat the mixture to dissolve the barbitone. Bring the volume up to 2.0 L with distilled water. The pH should be 7.4 - 7.6. Store at 4°C.

GVB²⁻. Dissolve 0.5 g of gelatin in 100 mL of distilled water and heat to dissolve. Add 100 mL of 5X VBS and make the total volume to 500 mL with distilled water. Store at 4°C. Make this buffer every few days.

D5W²⁻. Dissolve 25 g of D-glucose in 500 mL of distilled water. Make this solution every few days. Store at 4°C.

DGVB²⁺. Mix 250 mL of D5W with 250 mL of GVB²⁻. Add 75 µL of 1.0 M CaCl₂ and 100 µL of 2.5 M MgCl₂. Store at 4°C. Make this buffer every few days.

EDTA•GVB²⁻. Mix 1.489 g EDTA, 0.5 g gelatin, 100 mL of 5X VBS, and 200 mL of distilled water. Bring the pH up to 7.4 with 5M NaOH (this will also help to dissolve the EDTA). Make the volume up to 500 mL with distilled water. Store at 4°C. Make this buffer every few days.

EXAMPLE 1

This example illustrates the passive encapsulation of antisense oligonucleotides in liposomes. Additionally, this example illustrates that liposomes and unencapsulated DNA can be separated by their molecular weight difference using size exclusion chromatography.

1.1 Encapsulation of antisense

Egg phosphatidylcholine (EPC) (1.78 g) and cholesterol (CH) (0.72 g) were weighed out in a laminar flow hood and combined in chloroform in a 150 mL round bottomed flask. Chloroform was removed by roto-evaporation for 3 hours.

5 The antisense molecule ISIS 2302 (1.5 g) was dissolved in 10 mL of sterile phosphate buffered saline (PBS) and added to the lipid film composed of 2.5 g of EPC:CH (55:45; mol:mol). The resulting multilamellar vesicles (MLV) were transferred to three 5 mL cryovials and subjected to five cycles of freezing in liquid nitrogen and thawing at 40°C. The freeze-thawed MLV were combined, transferred into a sterile
10 100 mL capacity extruder (Lipex Biomembranes, Vancouver) and extruded ten times through one 0.1 µm filter.

Similar conditions were followed for smaller liposomal antisense preparations, except that 200 mg of oligonucleotide was rehydrated in 250 mg (total) of EPC:CH lipid.

1.2 DEAE-Sepharose Chromatography

15 Non-encapsulated (free) antisense molecule was separated from the entrapped molecule by anion exchange chromatography using DEAE-Sepharose CL-6B. In order to ensure reasonable sterility of the column material, the columns were washed with sterile PBS followed by elution of 300 mL of 100 nm vesicles composed of EPC
20 (24 mg/mL in PBS). Columns consisted of 6 mL syringes with a packed volume of 5 mL. A 400 mL aliquot of the antisense-liposome suspension was eluted on each column and the fractions containing lipid were collected and combined. Each column was used twice and then stored at 4°C for future recovery of the free antisense molecule.

The total volume of the column eluant was approximately 110 mL;
25 therefore, it was necessary to concentrate the liposomal antisense. The liposomal antisense was placed in dialysis tubing (MW cutoff, 14000) and concentrated to approximately 28 mL using Aquacide. Results of this purification are shown in Figure 2.

1.3 Measurement of Trapping Efficiency

30 Trapping efficiency was determined by size exclusion chromatography using a 1 mL Biogel A15m (fine) spin column as described previously (Chonn *et al.*,

1991). Briefly, a 50 μ L aliquot of the liposomal antisense was diluted with 70 μ L of PBS, and 50 μ L was eluted on each of two duplicate columns. Each fraction consisted of the volume eluted (\sim 40 μ L) during centrifugation at 1000 rpm for 1 min. The column eluant was collected, 50 μ L of PBS was added to the column, and the column was centrifuged again as described above, with the eluant being collected into a new 13 x 100 mm tube. This was repeated until a total of 36 fractions had been collected. Each fraction was assayed for lipid and DNA after the addition of 250 μ L of distilled water. Lipid was assayed by scintillation counting or phosphate analysis (Fiske and Subbarow, 1925) of a 50 μ L aliquot from each sample and the remainder (approximately 250 μ L) was assayed for DNA by a Bligh-Dyer extraction protocol. The results are shown in Figure 1.

1.4 Extraction Protocol

DNA in 250 μ L H₂O was separated from the lipid by the addition of 750 μ L of chloroform:methanol (1:2.1) to form a single phase consisting of chloroform:methanol:H₂O (1:2.1:1). Additional volumes of H₂O (250 μ L) and chloroform (250 μ L) were added to the sample, resulting in a two phase system. The sample was then centrifuged at 3000 rpm for 10 min to facilitate rapid separation of the organic and aqueous layers. The upper, aqueous, phase was collected and 400 μ L was assayed for DNA by absorbance at 260 nm.

The lower, chloroform, phase was washed three times with 300 μ L of methanol:H₂O (1:1) and dried under N₂. The lipid film was hydrated in 1 mL of H₂O and an aliquot was taken for phosphate assay.

1.5 Stability of the Encapsulated Liposomal Antisense

Since the liposomal antisense preparation was not always used immediately it was necessary to analyze the stability of the preparation over several days. To this end, retention of antisense in liposomes was measured over a 5 day period, at 4°C and at room temperature. Leakage of entrapped antisense would appear as a peak in the included volume of Biogel A15m spin columns. At various times, 50 μ L aliquots of the sample were applied to a 1 mL Biogel A15m column and eluted as described above. Lipid and oligonucleotide were determined as described above. A second peak in the oligonucleotide elution profile was taken to indicate leakage of entrapped oligonucleotide.

No leakage was indicated, either at 4°C or room temperature, even after 5 days (see Figures 3 and 4).

EXAMPLE 2

This example illustrates the complement activation by a liposomal antisense formulation.

To monitor the effects of various liposome and/or DNA formulations we have established an *in vitro* complement hemolytic assay using human serum for screening the relative complement activating properties of these formulations. The assay is a two-step procedure. The first step involves consumption of complement by liposomes and/or DNA, while the second step involves the lysis of antibody-sensitized sheep red cells by any residual complement that may not have been activated in the first part of the assay.

Complement activation by liposomes and free antisense was investigated using EPC/CH liposome compositions of 100 ± 30 nm diameter.

2.1 *Normal Human Serum Pool*

The first component of the assay that was tested was the activity of the fresh serum pool. This should be tested each time a new serum pool is generated as there will be some differences in complement activity between serum pools which can affect the sensitivity of the assay. A series of serum dilutions was tested to determine what dilution would give both maximal red cell lysis and minimal interference in absorbance readings (the more concentrated the serum dilution the more background absorbance is observed). Anything less than a 100-fold serum dilution gave reasonable levels of red cell lysis. EDTA-GVB²⁻ was added at the end of the assay to inhibit complement activity. The volume of EDTA-GVB²⁻ can be modified to increase or decrease the absorbance range of the assay depending on the activity of the serum pool. For all subsequent assays a 50-fold serum dilution was used (25-fold dilution in step 1 and 2-fold dilution in step 2). Furthermore, the amount of EDTA-GVB²⁻ used to stop the assay was 1.0 mL, giving an absorbance range (A_{410}) of 0 - 0.8.

Blood from seven healthy males and six healthy females was gathered into chilled serum tubes and immediately placed in an ice/water bath. Thirty mL of blood was collected per individual. Tubes were centrifuged at 2500 rpm for 10 min at 4°C, every six tubes (to avoid clotting). Plasma was removed from all tubes and pooled into a 250 mL beaker, on ice. The pooled plasma was then incubated at 37°C for 30 min, in the presence of several cloning sticks (to help recess the clot). The clot was removed and recessed, generating approximately 100 mL of serum. The serum was aliquoted (1.0 mL) into 1.5 mL Eppendorf tubes and stored at -65°C until use.

2.2 Preparation of Sensitized Sheep Red Cells (EA cells)

An aliquot of whole sheep blood (a 50% solution in Alsever's; Cedarlane) was withdrawn from the stock solution and centrifuged for 10 min at 1500 rpm. The cells were then washed three times with 10 volumes of EDTA-GVB²⁻. The washed cells were resuspended in a volume of EDTA-GVB²⁻ that is approximately 5-times that of the initial aliquot. An aliquot (100 µL) of the resuspended cells was mixed with 2.9 mL of distilled water in a cuvette and the absorbance at 541 nm was measured. The concentration of the cells was adjusted to 1×10^9 cells/mL with EDTA-GVB²⁻ according to the following information:

[RBC] (cells/mL)	O.D.	λ (nm)
1×10^9	0.385	541
5×10^8	0.192	541
2×10^8	0.654	414
1×10^8	0.327	414

The cell suspension was warmed to 37°C in a shaking bath and rabbit anti-sheep red blood cell antibody (hemolysin) was added to give a final antibody dilution of 1/500 (i.e. 20 µL of antibody into 10 mL of cells). This mixture was incubated for 30 min at 37°C. Following the incubation, the cells were centrifuged at 1500 rpm for 5 min at 4°C, the supernatant removed, and the cells washed with EDTA-GVB²⁻. The cells were then washed 2 times with DGVB²⁺ in order to further remove any free antibody and to introduce cations into the cell suspension. Finally, the cell concentration was adjusted to

2×10^8 cells/mL with DGVB²⁺ using the information given above. Cells were maintained at 4°C at all times, after preparation, and were used on the same day.

2.3 Complement Hemolytic Assay

All samples were serially diluted over a concentration range covering several orders of magnitude. Diluted liposome or oligonucleotide samples (100 µL) were added to 100 µL of normal human serum (NHS) that had been diluted 5-times in DGVB²⁺. Samples were incubated for 30 min at 37°C and subsequently placed on ice. Ice-cold DGVB²⁺ (300 µL) was added to each tube to dilute any complement not already consumed.

Samples (100 µL) of the diluted incubation mixture were added to 100 µL of EA cells or 100 µL of DGVB²⁺ (for color blanks - these are usually required at liposome concentrations > 1 mM). The mixture was incubated for 30 min at 37°C and subsequently placed on ice. EDTA-GVB²⁺ (1.0 mL) was added to the sample to inhibit complement activity and the mixture was centrifuged for 5 min at 4°C and 1500 rpm. Aliquots of the supernatant (250 µL) were transferred to a microtiter plate, in triplicate, with care not to disturb the pelleted red cells. The absorbance of the supernatant was measured at 410 nm on an electronic plate reader.

Figure 5 depicts the complement activating ability of the liposome composition. As can be seen, the neutral EPC:CH liposomes showed no observable complement activation over the concentration ranges studied.

EXAMPLE 3

This example illustrates the treatment of mice with liposomal antisense compositions to reduce inflammation due to delayed type hypersensitivity (DTH).

A murine model of contact sensitivity, a form of delayed type hypersensitivity (DTH), has been established. This model involves the sensitization of the abdominal region of mice with a strong contact sensitizing agent, dinitrofluorobenzene (DNFB). Inflammation can then be induced at a later time (5 days after initial sensitization) by challenging the ear with a dilute solution DNFB. This model has been characterized with respect to several common features of inflammation; specifically,

edema (ear thickness measurements), vascular leak (liposome accumulation in inflamed ears), and cell infiltration (myeloperoxidase assays for neutrophils/monocytes or by prelabeling bone marrow cells and circulating leukocytes with [³H]-thymidine). Furthermore, both inbred (BALB/c) and outbred (ICR) mice have been tested in this model, with similar patterns of inflammation being observed for both strains of mice.

3.1 Mice

Female BALB/c and ICR mice were obtained from Harley and Sprague Davis. BALB/c mice were used at 6-9 weeks of age, while ICR mice were used at 8-10 weeks of age. Each experimental group consists of four mice and the experiments were repeated at least twice.

3.2 Sensitization and Elicitation of Contact Sensitivity

Mice were sensitized by applying 25 μ L of 0.5% 2,4-dinitro-1-fluorobenzene (DNFB) in acetone:olive oil (4:1) to the shaved abdominal wall for two consecutive days. Four days after the second application, mice were challenged on the dorsal surface of the left ear with 10 μ L of 0.2% DNFB in acetone:olive oil (4:1). Mice received no treatment on the contralateral (right) ear. In some cases, control mice received 10 μ L of vehicle on the dorsal surface of the left ear.

3.3 Evaluation of Ear Swelling

Ear thickness was measured immediately prior to ear challenge, and at various time intervals after DNFB challenge, using an engineer's micrometer (Mitutoyo, Tokyo, Japan). Increases in ear thickness measurements were determined by subtracting the pre-challenge from post-challenge measurements.

The progression of ear inflammation over a 3 day period for ICR (outbred) and BALB/c (inbred) mice is indicated in Figures 6 and 7, respectively. Erythema was evident almost immediately after ear challenge and gradually declined in intensity over the remainder of the study. ICR mice exhibited peak ear thickness at 24 hours after the induction of ear inflammation. Maximal ear thickness measurements were found to be 170×10^{-4} inches, corresponding to a 70% increase in ear thickness. Although ear

swelling gradually declines at 48 and 72 hours after inflammation initiation, ear measurements still have not returned to baseline thickness levels ($90-100 \times 10^{-4}$ inches).

BALB/c mice demonstrate peak ear thickness measurements between 24-48 hours after ear challenge. The maximal ear thickness measurements exhibited by these mice were 130×10^{-4} inches, which corresponds to an increase of $\sim 50\%$ over baseline values ($75-85 \times 10^{-4}$ inches).

3.4 Evaluation of Liposome Accumulation

Large unilamellar vesicles composed of distearylphosphatidylcholine (DSPC) and cholesterol (CH), at a 55:45 molar ratio, were prepared in HEPES-buffered saline (20 mM HEPES, 145 mM NaCl, pH 7.4) by extrusion through 2 stacked 100 nm polycarbonate filters. Liposomes contained a non-exchangeable radioactive lipid marker, [^3H]cholesterylhexadecylether (CHE). LUVs were administered at a dose of 100 mg/kg (200 μL ; $\sim 2 \mu\text{Ci}$ of CHE/mouse) via the dorsal tail vein at 0, 24, 48, 72 hr after initiation of ear inflammation. Liposomes were allowed to circulate for 24 hr after taking ear measurements. Mice were then terminated and the ears were collected for analysis of liposome accumulation and cell infiltration.

Analyses of liposome accumulation were performed so as to give a relative indication of the degeneration of the vasculature during various stages of inflammation. Consequently, liposome accumulation was measured over the following time intervals: 0-24 hr, 24-48 hr, 48-72 hr (and in some cases 72-96 hr). For both strains of mice maximal liposome accumulation occurred during the first 24 hr (Figures 8 and 9). Thus, the most prominent changes in the vasculature likely occurred during the 0-24 hr time period.

3.5 Evaluation of Cell Infiltration

Cellular infiltration in inflamed and non-inflamed ears was assessed by an enzymatic assay for neutrophil (and monocyte) myeloperoxidase (MPO) activity, or by pre-labeling bone marrow cells and circulating leukocytes with [^3H]-methyl thymidine.

In order to extract MPO from the azurophilic granules of the cells rather harsh conditions were employed. Ears were finely minced with scissors and added to 2.0 mL of phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (HTAB) (50 mM potassium phosphate, 0.5% HTAB, pH 6.0). The sample was then homogenized

using a polytron homogenizer for 1 min (high output), and sonicated for 30 sec (power output, 4; 40% pulse). The sample was then divided into two 1.0 mL portions. One portion was digested, using Solvable, and analyzed for CHE by standard liquid scintillation counting. The second portion was freeze-thawed five times in liquid nitrogen and again sonicated for 30 sec (power output, 4; 40% pulse). The sample was then centrifuged for 10 min at 18 000 x g to remove cellular debris. The supernatant was removed and assayed for MPO activity by incubating 0.1 mL aliquots with 2.9 mL of substrate buffer (50 mM potassium phosphate, pH 6.0 containing 0.167 mg/mL o-dianisidine dihydrochloride and 0.0005% hydrogen peroxide). Absorbance at 460 nm was monitored for several minutes and, in most cases, ΔA_{460} was taken from the absorbances at 30 and 90 seconds. All solutions were maintained on ice as much as possible through the procedure. One unit of MPO activity is defined as the amount of enzyme that degrades 1 μ mol of peroxide per minute at 25°C.

For experiments in which blood cells were prelabeled with [3 H]-methyl thymidine, all mice received a 500 μ L i.p. injection [3 H]-methyl thymidine in sterile saline (1 μ Ci/g of body weight) 24 hours prior to challenging the ear with DNFB. Ears were removed after taking ear thickness measurements, digested with Solvable, and analyzed for labeled cells by standard liquid scintillation analysis. A relative estimate of cell infiltration was made by expressing the ratio of the radioactivity observed in the inflamed versus the non-inflamed ear. Figure 10 shows a time course MPO accumulation in inflamed versus non-inflamed ears. Peak MPO activity occurs at approximately 48 hr and returns to baseline levels by 96 hr. Based on the accumulation of radiolabeled blood cells (predominantly neutrophils, monocytes, and T-lymphocytes), cell accumulation peaks by 24 hr and remains relatively high over 72 hours (see Figure 11). The major difference in these two procedures is that the MPO assay primarily measures neutrophil accumulation (neutrophils have three times more MPO than monocytes), whereas the [3 H]-methyl thymidine procedure measures the influx of all cells equally. Neutrophil accumulation at sites of inflammation has been demonstrated to rise rapidly over the first 24 hours and to decrease almost as rapidly. From 24-48 hours, increased levels of monocytes and T-cells are observed at the inflammation site.

3.6 *A Typical Inflammation Experiment*

Figure 12 details a typical inflammation experiment involving ICR mice. Ear swelling begins to show increases at approximately 12 hr after ear challenge. Lipid accumulation begins almost immediately and consistently increases over 24 hr (the 6 hr, 12 hr, and 24 hr lipid accumulation time points in this figure were injected at $t=0$ hr). Finally, a lag period can be observed before the onset of cell infiltration, which begins to increase consistently after 6 hr, peaks at 24 hr, and then slowly declines over 72 hr.

EXAMPLE 4

This example illustrates the passive targeting of large unilamellar vesicles to sites of inflammation using a murine ear inflammation model.

Liposome accumulation appeared to be maximal during the 0-24 and 24-48 hr time periods after the onset of inflammation, corresponding to peak inflammatory events. After this, liposome accumulation decreased dramatically, corresponding to remodeling and repair of the "leaky" vasculature.

Of primary importance to the development of inflammation are the morphological and functional alterations that occur in dermal microvascular cells. When activated by cytokines, endothelial cells vasodilate, resulting in increased vascular blood flow to the region of inflammation. In addition, the blood vessels are stimulated to structurally remodel, thus enabling immune cells to extravasate from the vasculature and access the inflammation site. After such alterations, the endothelium is optimized for the infiltration of leukocytes and macromolecules to the site of inflammation. Consequently, it would be expected that relatively small vesicles, such as liposomes (100 nm diameter in our studies), would avidly move through the "leaky" vasculature and passively accumulate at sites of inflammation.

4.1 *Methods*

The mice used, as well as the sensitization and elicitation of contact sensitivity were carried out as described above in Example 3. Liposome accumulation was monitored over the first 24 hours of inflammation. LUVs were administered at a dose of 100 mg/kg (200 μ L; ~ 2 μ Ci of CHE/mouse) via the dorsal tail vein immediately

after ear challenge with DNFB. At 6, 12, and 24 hr after ear challenge, mice were terminated. Ears were collected and analyzed as described above.

4.2 *Liposome Accumulation at a Site of Inflammation*

Liposome accumulation was examined during various stages of murine ear inflammation so as to give a relative indication of the ability of these vesicles to extravasate through the "inflamed" vasculature. This is of interest for the passive targeting of liposomal drugs, such as anti-ICAM-1 oligonucleotides and corticosteroids, to sites of inflammation. Liposome accumulation was measured over the following time intervals: 0-24 hr, 24-48 hr, 48-72 hr. For both ICR and BALB/c mice, maximal liposome accumulation occurred during the first 24 hr of inflammation. Thus, it appears that the most prominent structural changes to the vasculature occurred during the 0-24 hr time period. This is consistent with previous reports detailing vascular leakage of relatively small molecules and proteins, such as BSA.

4.3 *Liposome Accumulation over the first 24 hr of Inflammation*

Figure 13 depicts the accumulation of DSPC:CH liposomes, administered immediately after ear challenge ($t=0$ hr), over the initial 24 hr of inflammation. As might be expected, lipid steadily accumulated in the inflamed ear during the first 24 hr, corresponding to the remodeling of the vascular endothelium in the inflamed region. No such increases in lipid accumulation were observed for control ears (non-inflamed), since no remodeling of the endothelium has occurred.

As Figure 13 illustrates, passive accumulation of LUVs occurs at sites of inflammation. Liposome accumulation appears to be maximal during the 0-24 hr time periods (after the onset of inflammation). After this, accumulation decreases dramatically, corresponding to remodeling and repair of the "leaky" vasculature.

EXAMPLE 5

This example illustrates the plasma clearance and biodistribution of free and encapsulated oligonucleotides.

Mice were sensitized and challenged as described in Example 3. Fifteen minutes after ear challenge, various antisense formulations were administered by the lateral tail (200 μ L) at an oligonucleotide dose of 50 mg/kg. Control mice were injected with PBS or saline. At various timepoints blood was withdrawn from the mice by cardiac puncture and collected into plasma tubes containing EDTA. An aliquot of whole blood was removed for analysis. The blood was centrifuged at 3000 rpm for 10 min and an aliquot of the plasma was counted by standard liquid scintillation analysis. Organs were collected, homogenized, digested and analyzed for the presence of radiolabeled antisense and radiolabeled lipid by standard liquid scintillation counting techniques.

The relative rates of clearance for free and encapsulated oligonucleotide are shown in Figure 14. The circulation half-life for free oligonucleotide (ISIS 2302) was very short (about 2.5 minutes). However, the formulation with antisense encapsulated in EPC:CH (55:45) liposomes had a much slower rate of clearance from the circulation. Instead of a circulation half-life of minutes, the encapsulated oligonucleotide had a half-life of about 8 hours. Thus, encapsulation increases the circulation half-life approximately 100-fold.

Figure 15 shows the tissue distribution of [3 H]-antisense in the liver, spleen, lung, and kidney after intravenous injection of oligonucleotide (50 mg/kg dose). The antisense molecule was rapidly cleared from the plasma with the majority of the dose distributing primarily to the liver and kidney. Only minor accumulation was observed in the spleen and lung. On the basis of organ weight, however, the kidney was the most efficient organ for antisense removal. This result is consistent with reports that indicate that the major route of elimination of free antisense from the body is by urinary excretion. Figure 16 demonstrates the biodistribution profiles for both the lipid and antisense portions of the encapsulated formulation. The liver was found to be the primary organ of accumulation for both antisense and lipid. Some accumulation was noted in the spleen with trace amounts being detected in the lungs and kidneys. This pattern of biodistribution is very similar to standard liposome biodistribution profiles but is significantly different from the kidney and liver tissue distribution exhibited by free oligonucleotide. Thus, the biodistribution of the lipid component determines the distribution of the antisense molecule as would be expected if the antisense molecule does not leak out of the liposomes.

EXAMPLE 6

This example illustrates the efficacy of mouse anti-ICAM oligonucleotide.

The efficacy of antisense oligonucleotide against mouse ICAM-1 mRNA was tested using the ear inflammation model described above (Example 3). The test oligonucleotide was developed by Isis Pharmaceuticals and is referred to as Isis 3082. The antisense is a 20 base (20 mer) phosphorothioate against a sequence in the untranslated 5'-region of murine ICAM-1 mRNA (see, Bennett, *et al.*, *J. Immunol.* 152:3530-3540 (1994); Bennett, *et al.*, *Adv. Pharmacol.* 28:1-43 (1994); and Chiang, *et al.*, *J. Biol. Chem.* 266:18162-18171 (1991)). This oligonucleotide has recently been shown to exhibit activity in reducing heart allograft rejection in mice (Stepkowski, *et al.*, *J. Immunol.* 153:5336-5346 (1994) and Stepkowski, *et al.*, *Transplant. Proc.* 27:113 (1995) but had not been found effective in the DTH model (personal communication, F. Bennett, Isis).

Ear thickness in the mice was measured immediately prior to ear challenge and 24 hr after DNFB challenge using an engineer's micrometer (Mitutoyo, Tokyo, Japan). Increases in ear thickness measurements were determined by subtracting the pre-challenge measurements from the post-challenge measurements.

Fifteen minutes after the initiation of murine ear inflammation, various antisense formulations were administered at an oligonucleotide dose of 50 mg/kg (in 200 μ L) via the dorsal tail vein. Inflammation control mice were injected with PBS. Ear measurements were taken 24 hours later to assess edema formation (ear swelling).

The effect of free anti-ICAM-1 oligonucleotides on the development of murine ear inflammation is indicated in Figure 17. As can be seen, each of the sets of mice (treated with either free ISIS 2302 or 3082) exhibited increased ear thickness measurements equivalent to the inflammation control mice which had been injected with PBS. Thus, a single dose (50 mg/kg) of free oligonucleotide was insufficient for preventing the development of ear edema.

Encapsulated antisense oligonucleotides were injected as described above and the results are shown in Figure 18. Increases in ear thickness were observed in mice

that received an injection of PBS. Similarly, mice treated with a single dose of free ISIS 3082 antisense, empty EPC:CH liposomes, or an encapsulated human ICAM-1 specific oligonucleotide (ISIS 2302) showed significant increases ($> 100\%$) in ear thickness. However, a murine ICAM-1 specific antisense molecule (ISI 3082) encapsulated in
5 EPC:CH liposomes was able to significantly reduce ear edema, whether injected 30 min prior to or immediately after initiating inflammation. This result is comparable to the control in which mice were treated topically with corticosteroid (HBP).

In a separate experiment, the ear weights of the DNFB-challenged and untreated ear were compared for mice treated with a single dose of free ISIS 3082,
10 encapsulated ISIS 3082, and PBS (see Figure 19). An approximate 2-fold increase in the weight of the challenged ear was observed for mice treated with either the free antisense molecule or PBS. However, little difference was observed between left and right ear weights of mice treated with encapsulated ISIS 3082, indicating a lack of edema.

All publications, patents and patent applications mentioned in this
15 specification are herein incorporated by reference into the specification to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that
20 certain changes and modifications may be practiced within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: University of British Columbia
- (B) STREET: 2194 Health Sciences Mall, Room IRC 331
- (C) CITY: Vancouver
- (D) STATE (PROVINCE): British Columbia
- (E) COUNTRY: Canada
- (F) POSTAL CODE: V6T 1Z3
- (G) TELEPHONE: (604) 822-8580
- (H) TELEFAX: (604) 822-8589
- (I) TELEX:

(ii) TITLE OF INVENTION: Enhanced Efficacy of Liposomal Antisense

(iii) NUMBER OF SEQUENCES: 10

(iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: Townsend and Townsend and Crew LLP
- (B) STREET: Two Embarcadero Center, Eighth Floor
- (C) CITY: San Francisco
- (D) STATE: California
- (E) COUNTRY: USA
- (F) ZIP: 94111-3834

(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

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(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Kezer, William B.
- (B) REGISTRATION NUMBER: 37,369
- (C) REFERENCE/DOCKET NUMBER: 16303-003600PC

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: (415) 576-0200
- (B) TELEFAX: (415) 576-0300

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2490 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 117..1949

(D) OTHER INFORMATION: /product= "human endothelial leukocyte
adhesion molecule 1 (ELAM-1)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CCAAAACGGA AAGTATTTCA AGCCTAAACC TTTGGGTGAA AAGAACTCTT GAAGTC	116
ATG ATT GCT TCA CAG TTT CTC TCA GCT CTC ACT TTG GTG CTT CTC ATT Met Ile Ala Ser Gln Phe Leu Ser Ala Leu Thr Leu Val Leu Leu Ile 1 5 10 15	164
AAA GAG AGT GGA GCC TGG TCT TAC AAC ACC TCC ACG GAA GCT ATG ACT Lys Glu Ser Gly Ala Trp Ser Tyr Asn Thr Ser Thr Glu Ala Met Thr 20 25 30	212
TAT GAT GAG GCC AGT GCT TAT TGT CAG CAA AGG TAC ACA CAC CTG GTT Tyr Asp Glu Ala Ser Ala Tyr Cys Gln Gln Arg Tyr Thr His Leu Val 35 40 45	260
GCA ATT CAA AAC AAA GAA GAG ATT GAG TAC CTA AAC TCC ATA TTG AGC Ala Ile Gln Asn Lys Glu Glu Ile Glu Tyr Leu Asn Ser Ile Leu Ser 50 55 60	308
TAT TCA CCA AGT TAT TAC TGG ATT GGA ATC AGA AAA GTC AAC AAT GTG Tyr Ser Pro Ser Tyr Tyr Trp Ile Gly Ile Arg Lys Val Asn Asn Val 65 70 75 80	356
TGG GTC TGG GTA GGA ACC CAG AAA CCT CTG ACA GAA GAA GCC AAG AAC Trp Val Trp Val Gly Thr Gln Lys Pro Leu Thr Glu Glu Ala Lys Asn 85 90 95	404
TGG GCT CCA GGT GAA CCC AAC AAT AGG CAA AAA GAT GAG GAC TGC GTG Trp Ala Pro Gly Glu Pro Asn Asn Arg Gln Lys Asp Glu Asp Cys Val 100 105 110	452
GAG ATC TAC ATC AAG AGA GAA AAA GAT GTG GGC ATG TGG AAT GAT GAG Glu Ile Tyr Ile Lys Arg Glu Lys Asp Val Gly Met Trp Asn Asp Glu 115 120 125	500
AGG TGC AGC AAG AAG AAG CTT GCC CTA TGC TAC ACA GCT GCC TGT ACC Arg Cys Ser Lys Lys Lys Leu Ala Leu Cys Tyr Thr Ala Ala Cys Thr 130 135 140	548
AAT ACA TCC TGC AGT GGC CAC GGT GAA TGT GTA GAG ACC ATC AAT AAT Asn Thr Ser Cys Ser Gly His Gly Glu Cys Val Glu Thr Ile Asn Asn 145 150 155 160	596
TAC ACT TGC AAG TGT GAC CCT GGC TTC AGT GGA CTC AAG TGT GAG CAA Tyr Thr Cys Lys Cys Asp Pro Gly Phe Ser Gly Leu Lys Cys Glu Gln 165 170 175	644
ATT GTG AAC TGT ACA GCC CTG GAA TCC CCT GAG CAT GGA AGC CTG GTT Ile Val Asn Cys Thr Ala Leu Glu Ser Pro Glu His Gly Ser Leu Val 180 185 190	692
TGC AGT CAC CCA CTG GGA AAC TTC AGC TAC AAT TCT TCC TGC TCT ATC Cys Ser His Pro Leu Gly Asn Phe Ser Tyr Asn Ser Ser Cys Ser Ile 195 200 205	740
AGC TGT GAT AGG GGT TAC CTG CCA AGC AGC ATG GAG ACC ATG CAG TGT Ser Cys Asp Arg Gly Tyr Leu Pro Ser Ser Met Glu Thr Met Gln Cys 210 215 220	788

43

ATG Met 225	TCC Ser	TCT Ser	GGA Gly	GAA Glu	TGG Trp	AGT Ser	GCT Ala	CCT Pro	ATT Ile	CCA Pro	GCC Ala	TGC Cys	AAT Asn	GTG Val	GTT Val	836
GAG Glu	TGT Cys	GAT Asp	GCT Ala	GTG Val	ACA Thr	AAT Asn	CCA Pro	GCC Ala	AAT Asn	GGG Gly	TTC Phe	GTG Val	GAA Glu	TGT Cys	TTC Phe	884
CAA Gln	AAC Asn	CCT Pro	GGA Gly	AGC Ser	TTC Phe	CCA Pro	TGG Trp	AAC Asn	ACA Thr	ACC Thr	TGT Cys	ACA Thr	TTT Phe	GAC Asp	TGT Cys	932
GAA Glu	GAA Glu	GGA Gly	TTT Phe	GAA Glu	CTA Leu	ATG Met	GGA Gly	GCC Ala	CAG Gln	AGC Ser	CTT Leu	CAG Gln	TGT Cys	ACC Thr	TCA Ser	980
TCT Ser	GGG Gly	AAT Asn	TGG Trp	GAC Asp	AAC Asn	GAG Glu	AAG Lys	CCA Pro	ACG Thr	TGT Cys	AAA Lys	GCT Ala	GTG Val	ACA Thr	TGC Cys	1028
AGG Arg	GCC Ala	GTC Val	CGC Arg	CAG Gln	CCT Pro	CAG Gln	AAT Asn	GGC Gly	TCT Ser	GTG Val	AGG Arg	TGC Cys	AGC Ser	CAT His	TCC Ser	1076
CCT Pro	GCT Ala	GGA Gly	GAG Glu	TTC Phe	ACC Thr	TTC Phe	AAA Lys	TCA Ser	TCC Ser	TGC Cys	AAC Asn	TTC Phe	ACC Thr	TGT Cys	GAG Glu	1124
GAA Glu	GGC Gly	TTC Phe	ATG Met	TTG Leu	CAG Gln	GGA Gly	CCA Pro	GCC Ala	CAG Gln	GTT Val	GAA Glu	TGC Cys	ACC Thr	ACT Thr	CAA Gln	1172
GGG Gly	CAG Gln	TGG Trp	ACA Thr	CAG Gln	CAA Gln	ATC Ile	CCA Pro	GTT Val	TGT Cys	GAA Glu	GCT Ala	TTC Phe	CAG Gln	TGC Cys	ACA Thr	1220
GCC Ala	TTG Leu	TCC Ser	AAC Asn	CCC Pro	GAG Glu	CGA Arg	GGC Gly	TAC Tyr	ATG Met	AAT Asn	TGT Cys	CTT Leu	CCT Pro	AGT Ser	GCT Ala	1268
TCT Ser	GGC Gly	AGT Ser	TTC Phe	CGT Arg	TAT Tyr	GGG Gly	TCC Ser	AGC Ser	TGT Cys	GAG Glu	TTC Phe	TCC Ser	TGT Cys	GAG Glu	CAG Gln	1316
GGT Gly	TTT Phe	GTG Val	TTG Leu	AAG Lys	GGA Gly	TCC Ser	AAA Lys	AGG Arg	CTC Leu	CAA Gln	TGT Cys	GGC Gly	CCC Pro	ACA Thr	GGG Gly	1364
GAG Glu	TGG Trp	GAC Asp	AAC Asn	GAG Glu	AAG Lys	CCC Pro	ACA Thr	TGT Cys	GAA Glu	GCT Ala	GTG Val	AGA Arg	TGC Cys	GAT Asp	GCT Ala	1412
GTC Val	CAC His	CAG Gln	CCC Pro	CCG Pro	AAG Lys	GGT Gly	TTG Leu	GTG Val	AGG Arg	TGT Cys	GCT Ala	CAT His	TCC Ser	CCT Pro	ATT Ile	1460
GGA Gly	GAA Glu	TTC Phe	ACC Thr	TAC Tyr	AAG Lys	TCC Ser	TCT Ser	TGT Cys	GCC Ala	TTC Phe	AGC Ser	TGT Cys	GAG Glu	GAG Glu	GGA Gly	1508
TTT Phe	GAA Glu	TTA Leu	TAT Tyr	GGA Gly	TCA Ser	ACT Thr	CAA Gln	CTT Leu	GAG Glu	TGC Cys	ACA Thr	TCT Ser	CAG Gln	GGA Gly	CAA Gln	1556
TGG Trp	ACA Thr	GAA Glu	GAG Glu	GTT Val	CCT Pro	TCC Ser	TGC Cys	CAA Gln	GTG Val	GTA Val	AAA Lys	TGT Cys	TCA Ser	AGC Ser	CTG Leu	1604

GCA GTT CCG GGA AAG ATC AAC ATG AGC TGC AGT GGG GAG CCC GTG TTT	1652
Ala Val Pro Gly Lys Ile Asn Met Ser Cys Ser Gly Glu Pro Val Phe	
500 505 510	
GGC ACT GTG TGC AAG TTC GCC TGT CCT GAA GGA TGG ACG CTC AAT GGC	1700
Gly Thr Val Cys Lys Phe Ala Cys Pro Glu Gly Trp Thr Leu Asn Gly	
515 520 525	
TCT GCA GCT CGG ACA TGT GGA GCC ACA GGA CAC TGG TCT GGC CTG CTA	1748
Ser Ala Ala Arg Thr Cys Gly Ala Thr Gly His Trp Ser Gly Leu Leu	
530 535 540	
CCT ACC TGT GAA GCT CCC ACT GAG TCC AAC ATT CCC TTG GTA GCT GGA	1796
Pro Thr Cys Glu Ala Pro Thr Glu Ser Asn Ile Pro Leu Val Ala Gly	
545 550 555 560	
CTT TCT GCT GCT GGA CTC TCC CTC CTG ACA TTA GCA CCA TTT CTC CTC	1844
Leu Ser Ala Ala Gly Leu Ser Leu Leu Thr Leu Ala Pro Phe Leu Leu	
565 570 575	
TGG CTT CGG AAA TGC TTA CGG AAA GCA AAG AAA TTT GTT CCT GCC AGC	1892
Trp Leu Arg Lys Cys Leu Arg Lys Ala Lys Lys Phe Val Pro Ala Ser	
580 585 590	
AGC TGC CAA AGC CTT GAA TCA GAC GGA AGC TAC CAA AAG CCT TCT TAC	1940
Ser Cys Gln Ser Leu Glu Ser Asp Gly Ser Tyr Gln Lys Pro Ser Tyr	
595 600 605	
ATC CTT TAAGTTCAAA AGAATCAGAA ACAGGTGCAT CTGGGGAAC AGAGGGATAC	1996
Ile Leu	
610	
ACTGAAGTTA ACAGAGACAG ATAACTCTCC TCGGGTCTCT GGCCCTTCTT GCCTACTATG	2056
CCAGATGCCT TTATGGCTGA AACCGCAACA CCCATCACCA CTTCAATAGA TCAAAGTCCA	2116
GCAGGCAAGG ACGGCCTTCA ACTGAAAAGA CTCAGTGTTC CCTTTCCTAC TCTCAGGATC	2176
AAGAAAGTGT TGGCTAATGA AGGGAAAGGA TATTTTCTTC CAAGCAAAGG TGAAGAGACC	2236
AAGACTCTGA AATCTCAGAA TTCCTTTTCT AACTCTCCCT TGCTCGCTGT AAAATCTTGG	2296
CACAGAAACA CAATATTTTG TGGCTTTCTT TCTTTTGCCC TTCACAGTGT TTCGACAGCT	2356
GATTACACAG TTGCTGTCAT AAGAATGAAT AATAATTATC CAGAGTTTAG AGGAAAAAAA	2416
TGACTAAAAA TATTATAACT TAAAAAATG ACAGATGTTG AATGCCACCA GGCAAATGCA	2476
TGGAGGGTTG TTAA	2490

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 610 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ile Ala Ser Gln Phe Leu Ser Ala Leu Thr Leu Val Leu Leu Ile
1 5 10 15

Lys Glu Ser Gly Ala Trp Ser Tyr Asn Thr Ser Thr Glu Ala Met Thr
20 25 30

45

Tyr Asp Glu Ala Ser Ala Tyr Cys Gln Gln Arg Tyr Thr His Leu Val
 35 40 45
 Ala Ile Gln Asn Lys Glu Glu Ile Glu Tyr Leu Asn Ser Ile Leu Ser
 50 55 60
 Tyr Ser Pro Ser Tyr Tyr Trp Ile Gly Ile Arg Lys Val Asn Asn Val
 65 70 75 80
 Trp Val Trp Val Gly Thr Gln Lys Pro Leu Thr Glu Glu Ala Lys Asn
 85 90 95
 Trp Ala Pro Gly Glu Pro Asn Asn Arg Gln Lys Asp Glu Asp Cys Val
 100 105 110
 Glu Ile Tyr Ile Lys Arg Glu Lys Asp Val Gly Met Trp Asn Asp Glu
 115 120 125
 Arg Cys Ser Lys Lys Lys Leu Ala Leu Cys Tyr Thr Ala Ala Cys Thr
 130 135 140
 Asn Thr Ser Cys Ser Gly His Gly Glu Cys Val Glu Thr Ile Asn Asn
 145 150 155 160
 Tyr Thr Cys Lys Cys Asp Pro Gly Phe Ser Gly Leu Lys Cys Glu Gln
 165 170 175
 Ile Val Asn Cys Thr Ala Leu Glu Ser Pro Glu His Gly Ser Leu Val
 180 185 190
 Cys Ser His Pro Leu Gly Asn Phe Ser Tyr Asn Ser Ser Cys Ser Ile
 195 200 205
 Ser Cys Asp Arg Gly Tyr Leu Pro Ser Ser Met Glu Thr Met Gln Cys
 210 215 220
 Met Ser Ser Gly Glu Trp Ser Ala Pro Ile Pro Ala Cys Asn Val Val
 225 230 235 240
 Glu Cys Asp Ala Val Thr Asn Pro Ala Asn Gly Phe Val Glu Cys Phe
 245 250 255
 Gln Asn Pro Gly Ser Phe Pro Trp Asn Thr Thr Cys Thr Phe Asp Cys
 260 265 270
 Glu Glu Gly Phe Glu Leu Met Gly Ala Gln Ser Leu Gln Cys Thr Ser
 275 280 285
 Ser Gly Asn Trp Asp Asn Glu Lys Pro Thr Cys Lys Ala Val Thr Cys
 290 295 300
 Arg Ala Val Arg Gln Pro Gln Asn Gly Ser Val Arg Cys Ser His Ser
 305 310 315 320
 Pro Ala Gly Glu Phe Thr Phe Lys Ser Ser Cys Asn Phe Thr Cys Glu
 325 330 335
 Glu Gly Phe Met Leu Gln Gly Pro Ala Gln Val Glu Cys Thr Thr Gln
 340 345 350
 Gly Gln Trp Thr Gln Gln Ile Pro Val Cys Glu Ala Phe Gln Cys Thr
 355 360 365
 Ala Leu Ser Asn Pro Glu Arg Gly Tyr Met Asn Cys Leu Pro Ser Ala
 370 375 380

46

Ser Gly Ser Phe Arg Tyr Gly Ser Ser Cys Glu Phe Ser Cys Glu Gln
 385 390 395 400
 Gly Phe Val Leu Lys Gly Ser Lys Arg Leu Gln Cys Gly Pro Thr Gly
 405 410 415
 Glu Trp Asp Asn Glu Lys Pro Thr Cys Glu Ala Val Arg Cys Asp Ala
 420 425 430
 Val His Gln Pro Pro Lys Gly Leu Val Arg Cys Ala His Ser Pro Ile
 435 440 445
 Gly Glu Phe Thr Tyr Lys Ser Ser Cys Ala Phe Ser Cys Glu Glu Gly
 450 455 460
 Phe Glu Leu Tyr Gly Ser Thr Gln Leu Glu Cys Thr Ser Gln Gly Gln
 465 470 475 480
 Trp Thr Glu Glu Val Pro Ser Cys Gln Val Val Lys Cys Ser Ser Leu
 485 490 495
 Ala Val Pro Gly Lys Ile Asn Met Ser Cys Ser Gly Glu Pro Val Phe
 500 505 510
 Gly Thr Val Cys Lys Phe Ala Cys Pro Glu Gly Trp Thr Leu Asn Gly
 515 520 525
 Ser Ala Ala Arg Thr Cys Gly Ala Thr Gly His Trp Ser Gly Leu Leu
 530 535 540
 Pro Thr Cys Glu Ala Pro Thr Glu Ser Asn Ile Pro Leu Val Ala Gly
 545 550 555 560
 Leu Ser Ala Ala Gly Leu Ser Leu Leu Thr Leu Ala Pro Phe Leu Leu
 565 570 575
 Trp Leu Arg Lys Cys Leu Arg Lys Ala Lys Lys Phe Val Pro Ala Ser
 580 585 590
 Ser Cys Gln Ser Leu Glu Ser Asp Gly Ser Tyr Gln Lys Pro Ser Tyr
 595 600 605
 Ile Leu
 610

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2220 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..2220
- (D) OTHER INFORMATION: /product= "human vascular cell adhesion molecule 1 (VCAM-1)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATG	CCT	GGG	AAG	ATG	GTC	GTG	ATC	CTT	GGA	GCC	TCA	AAT	ATA	CTT	TGG	48
Met	Pro	Gly	Lys	Met	Val	Val	Ile	Leu	Gly	Ala	Ser	Asn	Ile	Leu	Trp	
1				5				10						15		
ATA	ATG	TTT	GCA	GCT	TCT	CAA	GCT	TTT	AAA	ATC	GAG	ACC	ACC	CCA	GAA	96
Ile	Met	Phe	Ala	Ala	Ser	Gln	Ala	Phe	Lys	Ile	Glu	Thr	Thr	Pro	Glu	
			20					25					30			
TCT	AGA	TAT	CTT	GCT	CAG	ATT	GGT	GAC	TCC	GTC	TCA	TTG	ACT	TGC	AGC	144
Ser	Arg	Tyr	Leu	Ala	Gln	Ile	Gly	Asp	Ser	Val	Ser	Leu	Thr	Cys	Ser	
			35				40					45				
ACC	ACA	GGC	TGT	GAG	TCC	CCA	TTT	TTC	TCT	TGG	AGA	ACC	CAG	ATA	GAT	192
Thr	Thr	Gly	Cys	Glu	Ser	Pro	Phe	Phe	Ser	Trp	Arg	Thr	Gln	Ile	Asp	
	50					55					60					
AGT	CCA	CTG	AAT	GGG	AAG	GTG	ACG	AAT	GAG	GGG	ACC	ACA	TCT	ACG	CTG	240
Ser	Pro	Leu	Asn	Gly	Lys	Val	Thr	Asn	Glu	Gly	Thr	Thr	Ser	Thr	Leu	
65				70					75						80	
ACA	ATG	AAT	CCT	GTT	AGT	TTT	GGG	AAC	GAA	CAC	TCT	TAC	CTG	TGC	ACA	288
Thr	Met	Asn	Pro	Val	Ser	Phe	Gly	Asn	Glu	His	Ser	Tyr	Leu	Cys	Thr	
			85					90						95		
GCA	ACT	TGT	GAA	TCT	AGG	AAA	TTG	GAA	AAA	GGA	ATC	CAG	GTG	GAG	ATC	336
Ala	Thr	Cys	Glu	Ser	Arg	Lys	Leu	Glu	Lys	Gly	Ile	Gln	Val	Glu	Ile	
			100					105					110			
TAC	TCT	TTT	CCT	AAG	GAT	CCA	GAG	ATT	CAT	TTG	AGT	GGC	CCT	CTG	GAG	384
Tyr	Ser	Phe	Pro	Lys	Asp	Pro	Glu	Ile	His	Leu	Ser	Gly	Pro	Leu	Glu	
			115				120					125				
GCT	GGG	AAG	CCG	ATC	ACA	GTC	AAG	TGT	TCA	GTT	GCT	GAT	GTA	TAC	CCA	432
Ala	Gly	Lys	Pro	Ile	Thr	Val	Lys	Cys	Ser	Val	Ala	Asp	Val	Tyr	Pro	
	130					135					140					
TTT	GAC	AGG	CTG	GAG	ATA	GAC	TTA	CTG	AAA	GGA	GAT	CAT	CTC	ATG	AAG	480
Phe	Asp	Arg	Leu	Glu	Ile	Asp	Leu	Leu	Lys	Gly	Asp	His	Leu	Met	Lys	
145				150					155					160		
AGT	CAG	GAA	TTT	CTG	GAG	GAT	GCA	GAC	AGG	AAG	TCC	CTG	GAA	ACC	AAG	528
Ser	Gln	Glu	Phe	Leu	Glu	Asp	Ala	Asp	Arg	Lys	Ser	Leu	Glu	Thr	Lys	
			165					170						175		
AGT	TTG	GAA	GTA	ACC	TTT	ACT	CCT	GTC	ATT	GAG	GAT	ATT	GGA	AAA	GTT	576
Ser	Leu	Glu	Val	Thr	Phe	Thr	Pro	Val	Ile	Glu	Asp	Ile	Gly	Lys	Val	
			180					185					190			
CTT	GTT	TGC	CGA	GCT	AAA	TTA	CAC	ATT	GAT	GAA	ATG	GAT	TCT	GTG	CCC	624
Leu	Val	Cys	Arg	Ala	Lys	Leu	His	Ile	Asp	Glu	Met	Asp	Ser	Val	Pro	
			195				200					205				
ACA	GTA	AGG	CAG	GCT	GTA	AAA	GAA	TTG	CAA	GTC	TAC	ATA	TCA	CCC	AAG	672
Thr	Val	Arg	Gln	Ala	Val	Lys	Glu	Leu	Gln	Val	Tyr	Ile	Ser	Pro	Lys	
	210					215					220					
AAT	ACA	GTT	ATT	TCT	GTG	AAT	CCA	TCC	ACA	AAG	CTG	CAA	GAA	GGT	GGC	720
Asn	Thr	Val	Ile	Ser	Val	Asn	Pro	Ser	Thr	Lys	Leu	Gln	Glu	Gly	Gly	
225				230					235					240		
TCT	GTG	ACC	ATG	ACC	TGT	TCC	AGC	GAG	GGT	CTA	CCA	GCT	CCA	GAG	ATT	768
Ser	Val	Thr	Met	Thr	Cys	Ser	Ser	Glu	Gly	Leu	Pro	Ala	Pro	Glu	Ile	
				245				250						255		

TTC TGG AGT AAG AAA TTA GAT AAT GGG AAT CTA CAG CAC CTT TCT GGA	816
Phe Trp Ser Lys Lys Leu Asp Asn Gly Asn Leu Gln His Leu Ser Gly	
260 265 270	
AAT GCA ACT CTC ACC TTA ATT GCT ATG AGG ATG GAA GAT TCT GGA ATT	864
Asn Ala Thr Leu Thr Leu Ile Ala Met Arg Met Glu Asp Ser Gly Ile	
275 280 285	
TAT GTG TGT GAA GGA GTT AAT TTG ATT GGG AAA AAC AGA AAA GAG GTG	912
Tyr Val Cys Glu Gly Val Asn Leu Ile Gly Lys Asn Arg Lys Glu Val	
290 295 300	
GAA TTA ATT GTT CAA GAG AAA CCA TTT ACT GTT GAG ATC TCC CCT GGA	960
Glu Leu Ile Val Gln Glu Lys Pro Phe Thr Val Glu Ile Ser Pro Gly	
305 310 315 320	
CCC CGG ATT GCT GCT CAG ATT GGA GAC TCA GTC ATG TTG ACA TGT AGT	1008
Pro Arg Ile Ala Ala Gln Ile Gly Asp Ser Val Met Leu Thr Cys Ser	
325 330 335	
GTC ATG GGC TGT GAA TCC CCA TCT TTC TCC TGG AGA ACC CAG ATA GAC	1056
Val Met Gly Cys Glu Ser Pro Ser Phe Ser Trp Arg Thr Gln Ile Asp	
340 345 350	
AGC CCT CTG AGC GGG AAG GTG AGG AGT GAG GGG ACC AAT TCC ACG CTG	1104
Ser Pro Leu Ser Gly Lys Val Arg Ser Glu Gly Thr Asn Ser Thr Leu	
355 360 365	
ACC CTG AGC CCT GTG AGT TTT GAG AAC GAA CAC TCT TAT CTG TGC ACA	1152
Thr Leu Ser Pro Val Ser Phe Glu Asn Glu His Ser Tyr Leu Cys Thr	
370 375 380	
GTG ACT TGT GGA CAT AAG AAA CTG GAA AAG GGA ATC CAG GTG GAG CTC	1200
Val Thr Cys Gly His Lys Lys Leu Glu Lys Gly Ile Gln Val Glu Leu	
385 390 395 400	
TAC TCA TTC CCT AGA GAT CCA GAA ATC GAG ATG AGT GGT GGC CTC GTG	1248
Tyr Ser Phe Pro Arg Asp Pro Glu Ile Glu Met Ser Gly Gly Leu Val	
405 410 415	
AAT GGG AGC TCT GTC ACT GTA AGC TGC AAG GTT CCT AGC GTG TAC CCC	1296
Asn Gly Ser Ser Val Thr Val Ser Cys Lys Val Pro Ser Val Tyr Pro	
420 425 430	
CTT GAC CGG CTG GAG ATT GAA TTA CTT AAG GGG GAG ACT ATT CTG GAG	1344
Leu Asp Arg Leu Glu Ile Glu Leu Lys Gly Glu Thr Ile Leu Glu	
435 440 445	
AAT ATA GAG TTT TTG GAG GAT ACG GAT ATG AAA TCT CTA GAG AAC AAA	1392
Asn Ile Glu Phe Leu Glu Asp Thr Asp Met Lys Ser Leu Glu Asn Lys	
450 455 460	
AGT TTG GAA ATG ACC TTC ATC CCT ACC ATT GAA GAT ACT GGA AAA GCT	1440
Ser Leu Glu Met Thr Phe Ile Pro Thr Ile Glu Asp Thr Gly Lys Ala	
465 470 475 480	
CTT GTT TGT CAG GCT AAG TTA CAT ATT GAT GAC ATG GAA TTC GAA CCC	1488
Leu Val Cys Gln Ala Lys Leu His Ile Asp Asp Met Glu Phe Glu Pro	
485 490 495	
AAA CAA AGG CAG AGT ACG CAA ACA CTT TAT GTC AAT GTT GCC CCC AGA	1536
Lys Gln Arg Gln Ser Thr Gln Thr Leu Tyr Val Asn Val Ala Pro Arg	
500 505 510	
GAT ACA ACC GTC TTG GTC AGC CCT TCC TCC ATC CTG GAG GAA GGC AGT	1584
Asp Thr Thr Val Leu Val Ser Pro Ser Ser Ile Leu Glu Glu Gly Ser	
515 520 525	

49

TCT	GTG	AAT	ATG	ACA	TGC	TTG	AGC	CAG	GGC	TTT	CCT	GCT	CCG	AAA	ATC	1632
Ser	Val	Asn	Met	Thr	Cys	Leu	Ser	Gln	Gly	Phe	Pro	Ala	Pro	Lys	Ile	
530						535					540					
CTG	TGG	AGC	AGG	CAG	CTC	CCT	AAC	GGG	GAG	CTA	CAG	CCT	CTT	TCT	GAG	1680
Leu	Trp	Ser	Arg	Gln	Leu	Pro	Asn	Gly	Glu	Leu	Gln	Pro	Leu	Ser	Glu	
545					550					555					560	
AAT	GCA	ACT	CTC	ACC	TTA	ATT	TCT	ACA	AAA	ATG	GAA	GAT	TCT	GGG	GTT	1728
Asn	Ala	Thr	Leu	Thr	Leu	Ile	Ser	Thr	Lys	Met	Glu	Asp	Ser	Gly	Val	
				565					570					575		
TAT	TTA	TGT	GAA	GGA	ATT	AAC	CAG	GCT	GGA	AGA	AGC	AGA	AAG	GAA	GTG	1776
Tyr	Leu	Cys	Glu	Gly	Ile	Asn	Gln	Ala	Gly	Arg	Ser	Arg	Lys	Glu	Val	
			580					585					590			
GAA	TTA	ATT	ATC	CAA	GTT	ACT	CCA	AAA	GAC	ATA	AAA	CTT	ACA	GCT	TTT	1824
Glu	Leu	Ile	Ile	Gln	Val	Thr	Pro	Lys	Asp	Ile	Lys	Leu	Thr	Ala	Phe	
			595				600					605				
CCT	TCT	GAG	AGT	GTC	AAA	GAA	GGA	GAC	ACT	GTC	ATC	ATC	TCT	TGT	ACA	1872
Pro	Ser	Glu	Ser	Val	Lys	Glu	Gly	Asp	Thr	Val	Ile	Ile	Ser	Cys	Thr	
	610					615					620					
TGT	GGA	AAT	GTT	CCA	GAA	ACA	TGG	ATA	ATC	CTG	AAG	AAA	AAA	GCG	GAG	1920
Cys	Gly	Asn	Val	Pro	Glu	Thr	Trp	Ile	Ile	Leu	Lys	Lys	Lys	Ala	Glu	
625					630					635					640	
ACA	GGA	GAC	ACA	GTA	CTA	AAA	TCT	ATA	GAT	GGC	GCC	TAT	ACC	ATC	CGA	1968
Thr	Gly	Asp	Thr	Val	Leu	Lys	Ser	Ile	Asp	Gly	Ala	Tyr	Thr	Ile	Arg	
				645					650					655		
AAG	GCC	CAG	TTG	AAG	GAT	GCG	GGA	GTA	TAT	GAA	TGT	GAA	TCT	AAA	AAC	2016
Lys	Ala	Gln	Leu	Lys	Asp	Ala	Gly	Val	Tyr	Glu	Cys	Glu	Ser	Lys	Asn	
			660				665						670			
AAA	GTT	GGC	TCA	CAA	TTA	AGA	AGT	TTA	ACA	CTT	GAT	GTT	CAA	GGA	AGA	2064
Lys	Val	Gly	Ser	Gln	Leu	Arg	Ser	Leu	Thr	Leu	Asp	Val	Gln	Gly	Arg	
			675				680					685				
GAA	AAC	AAC	AAA	GAC	TAT	TTT	TCT	CCT	GAG	CTT	CTC	GTG	CTC	TAT	TTT	2112
Glu	Asn	Asn	Lys	Asp	Tyr	Phe	Ser	Pro	Glu	Leu	Val	Leu	Tyr	Phe		
	690					695					700					
GCA	TCC	TCC	TTA	ATA	ATA	CCT	GCC	ATT	GGA	ATG	ATA	ATT	TAC	TTT	GCA	2160
Ala	Ser	Ser	Leu	Ile	Ile	Pro	Ala	Ile	Gly	Met	Ile	Ile	Tyr	Phe	Ala	
705					710					715					720	
AGA	AAA	GCC	AAC	ATG	AAG	GGG	TCA	TAT	AGT	CTT	GTA	GAA	GCA	CAG	AAA	2208
Arg	Lys	Ala	Asn	Met	Lys	Gly	Ser	Tyr	Ser	Leu	Val	Glu	Ala	Gln	Lys	
				725					730					735		
TCA	AAA	GTG	TAG													2220
Ser	Lys	Val														

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 739 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Pro Gly Lys Met Val Val Ile Leu Gly Ala Ser Asn Ile Leu Trp
 1 5 10 15
 Ile Met Phe Ala Ala Ser Gln Ala Phe Lys Ile Glu Thr Thr Pro Glu
 20 25 30
 Ser Arg Tyr Leu Ala Gln Ile Gly Asp Ser Val Ser Leu Thr Cys Ser
 35 40 45
 Thr Thr Gly Cys Glu Ser Pro Phe Phe Ser Trp Arg Thr Gln Ile Asp
 50 55 60
 Ser Pro Leu Asn Gly Lys Val Thr Asn Glu Gly Thr Thr Ser Thr Leu
 65 70 75 80
 Thr Met Asn Pro Val Ser Phe Gly Asn Glu His Ser Tyr Leu Cys Thr
 85 90 95
 Ala Thr Cys Glu Ser Arg Lys Leu Glu Lys Gly Ile Gln Val Glu Ile
 100 105 110
 Tyr Ser Phe Pro Lys Asp Pro Glu Ile His Leu Ser Gly Pro Leu Glu
 115 120 125
 Ala Gly Lys Pro Ile Thr Val Lys Cys Ser Val Ala Asp Val Tyr Pro
 130 135 140
 Phe Asp Arg Leu Glu Ile Asp Leu Leu Lys Gly Asp His Leu Met Lys
 145 150 155 160
 Ser Gln Glu Phe Leu Glu Asp Ala Asp Arg Lys Ser Leu Glu Thr Lys
 165 170 175
 Ser Leu Glu Val Thr Phe Thr Pro Val Ile Glu Asp Ile Gly Lys Val
 180 185 190
 Leu Val Cys Arg Ala Lys Leu His Ile Asp Glu Met Asp Ser Val Pro
 195 200 205
 Thr Val Arg Gln Ala Val Lys Glu Leu Gln Val Tyr Ile Ser Pro Lys
 210 215 220
 Asn Thr Val Ile Ser Val Asn Pro Ser Thr Lys Leu Gln Glu Gly Gly
 225 230 235 240
 Ser Val Thr Met Thr Cys Ser Ser Glu Gly Leu Pro Ala Pro Glu Ile
 245 250 255
 Phe Trp Ser Lys Lys Leu Asp Asn Gly Asn Leu Gln His Leu Ser Gly
 260 265 270
 Asn Ala Thr Leu Thr Leu Ile Ala Met Arg Met Glu Asp Ser Gly Ile
 275 280 285
 Tyr Val Cys Glu Gly Val Asn Leu Ile Gly Lys Asn Arg Lys Glu Val
 290 295 300
 Glu Leu Ile Val Gln Glu Lys Pro Phe Thr Val Glu Ile Ser Pro Gly
 305 310 315 320
 Pro Arg Ile Ala Ala Gln Ile Gly Asp Ser Val Met Leu Thr Cys Ser
 325 330 335
 Val Met Gly Cys Glu Ser Pro Ser Phe Ser Trp Arg Thr Gln Ile Asp
 340 345 350

Ser Pro Leu Ser Gly Lys Val Arg Ser Glu Gly Thr Asn Ser Thr Leu
 355 360 365
 Thr Leu Ser Pro Val Ser Phe Glu Asn Glu His Ser Tyr Leu Cys Thr
 370 375 380
 Val Thr Cys Gly His Lys Lys Leu Glu Lys Gly Ile Gln Val Glu Leu
 385 390 395 400
 Tyr Ser Phe Pro Arg Asp Pro Glu Ile Glu Met Ser Gly Gly Leu Val
 405 410 415
 Asn Gly Ser Ser Val Thr Val Ser Cys Lys Val Pro Ser Val Tyr Pro
 420 425 430
 Leu Asp Arg Leu Glu Ile Glu Leu Leu Lys Gly Glu Thr Ile Leu Glu
 435 440 445
 Asn Ile Glu Phe Leu Glu Asp Thr Asp Met Lys Ser Leu Glu Asn Lys
 450 455 460
 Ser Leu Glu Met Thr Phe Ile Pro Thr Ile Glu Asp Thr Gly Lys Ala
 465 470 475 480
 Leu Val Cys Gln Ala Lys Leu His Ile Asp Asp Met Glu Phe Glu Pro
 485 490 495
 Lys Gln Arg Gln Ser Thr Gln Thr Leu Tyr Val Asn Val Ala Pro Arg
 500 505 510
 Asp Thr Thr Val Leu Val Ser Pro Ser Ser Ile Leu Glu Glu Gly Ser
 515 520 525
 Ser Val Asn Met Thr Cys Leu Ser Gln Gly Phe Pro Ala Pro Lys Ile
 530 535 540
 Leu Trp Ser Arg Gln Leu Pro Asn Gly Glu Leu Gln Pro Leu Ser Glu
 545 550 555 560
 Asn Ala Thr Leu Thr Leu Ile Ser Thr Lys Met Glu Asp Ser Gly Val
 565 570 575
 Tyr Leu Cys Glu Gly Ile Asn Gln Ala Gly Arg Ser Arg Lys Glu Val
 580 585 590
 Glu Leu Ile Ile Gln Val Thr Pro Lys Asp Ile Lys Leu Thr Ala Phe
 595 600 605
 Pro Ser Glu Ser Val Lys Glu Gly Asp Thr Val Ile Ile Ser Cys Thr
 610 615 620
 Cys Gly Asn Val Pro Glu Thr Trp Ile Ile Leu Lys Lys Lys Ala Glu
 625 630 635 640
 Thr Gly Asp Thr Val Leu Lys Ser Ile Asp Gly Ala Tyr Thr Ile Arg
 645 650 655
 Lys Ala Gln Leu Lys Asp Ala Gly Val Tyr Glu Cys Glu Ser Lys Asn
 660 665 670
 Lys Val Gly Ser Gln Leu Arg Ser Leu Thr Leu Asp Val Gln Gly Arg
 675 680 685
 Glu Asn Asn Lys Asp Tyr Phe Ser Pro Glu Leu Leu Val Leu Tyr Phe
 690 695 700

Ala Ser Ser Leu Ile Ile Pro Ala Ile Gly Met Ile Ile Tyr Phe Ala
 705 710 715 720

Arg Lys Ala Asn Met Lys Gly Ser Tyr Ser Leu Val Glu Ala Gln Lys
 725 730 735

Ser Lys Val

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1846 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 13..1611
- (D) OTHER INFORMATION: /product= "human intercellular adhesion molecule 1 (ICAM-1)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTCAGCCTCG CT ATG GCT CCC AGC AGC CCC CGG CCC GCG CTG CCC GCA	48
Met Ala Pro Ser Ser Pro Arg Pro Ala Leu Pro Ala	
1 5 10	
CTC CTG GTC CTG CTC GGG GCT CTG TTC CCA GGA CCT GGC AAT GCC CAG	96
Leu Leu Val Leu Leu Gly Ala Leu Phe Pro Gly Pro Gly Asn Ala Gln	
15 20 25	
ACA TCT GTG TCC CCC TCA AAA GTC ATC CTG CCC CGG GGA GGC TCC GTG	144
Thr Ser Val Ser Pro Ser Lys Val Ile Leu Pro Arg Gly Gly Ser Val	
30 35 40	
CTG GTG ACA TGC AGC ACC TCC TGT GAC CAG CCC AAG TTG TTG GGC ATA	192
Leu Val Thr Cys Ser Thr Ser Cys Asp Gln Pro Lys Leu Leu Gly Ile	
45 50 55 60	
GAG ACC CCG TTG CCT AAA AAG GAG TTG CTC CTG CCT GGG AAC AAC CGG	240
Glu Thr Pro Leu Pro Lys Lys Glu Leu Leu Leu Pro Gly Asn Asn Arg	
65 70 75	
AAG GTG TAT GAA CTG AGC AAT GTG CAA GAA GAT AGC CAA CCA ATG TGC	288
Lys Val Tyr Glu Leu Ser Asn Val Gln Glu Asp Ser Gln Pro Met Cys	
80 85 90	
TAT TCA AAC TGC CCT GAT GGG CAG TCA ACA GCT AAA ACC TTC CTC ACC	336
Tyr Ser Asn Cys Pro Asp Gly Gln Ser Thr Ala Lys Thr Phe Leu Thr	
95 100 105	
GTG TAC TGG ACT CCA GAA CGG GTG GAA CTG GCA CCC CTC CCC TCT TGG	384
Val Tyr Trp Thr Pro Glu Arg Val Glu Leu Ala Pro Leu Pro Ser Trp	
110 115 120	
CAG CCA GTG GGC AAG AAC CTT ACC CTA CGC TGC CAG GTG GAG GGT GGG	432
Gln Pro Val Gly Lys Asn Leu Thr Leu Arg Cys Gln Val Glu Gly Gly	
125 130 135 140	

53

GCA	CCC	CGG	GCC	AAC	CTC	ACC	GTG	GTG	CTG	CTC	CGT	GGG	GAG	AAG	GAG	480
Ala	Pro	Arg	Ala	Asn	Leu	Thr	Val	Val	Leu	Leu	Arg	Gly	Glu	Lys	Glu	
				145					150					155		
CTG	AAA	CGG	GAG	CCA	GCT	GTG	GGG	GAG	CCC	GCT	GAG	GTC	ACG	ACC	ACG	528
Leu	Lys	Arg	Glu	Pro	Ala	Val	Gly	Glu	Pro	Ala	Glu	Val	Thr	Thr	Thr	
			160					165					170			
GTG	CTG	GTG	AGG	AGA	GAT	CAC	CAT	GGA	GCC	AAT	TTC	TCG	TGC	CGC	ACT	576
Val	Leu	Val	Arg	Arg	Asp	His	His	Gly	Ala	Asn	Phe	Ser	Cys	Arg	Thr	
			175				180						185			
GAA	CTG	GAC	CTG	CGG	CCC	CAA	GGG	CTG	GAG	CTG	TTT	GAG	AAC	ACC	TCG	624
Glu	Leu	Asp	Leu	Arg	Pro	Gln	Gly	Leu	Glu	Leu	Phe	Glu	Asn	Thr	Ser	
	190					195					200					
GCC	CCC	TAC	CAG	CTC	CAG	ACC	TTT	GTC	CTG	CCA	GCG	ACT	CCC	CCA	CAA	672
Ala	Pro	Tyr	Gln	Leu	Gln	Thr	Phe	Val	Leu	Pro	Ala	Thr	Pro	Pro	Gln	
	205				210					215					220	
CTT	GTC	AGC	CCC	CGG	GTC	CTA	GAG	GTG	GAC	ACG	CAG	GGG	ACC	GTG	GTC	720
Leu	Val	Ser	Pro	Arg	Val	Leu	Glu	Val	Asp	Thr	Gln	Gly	Thr	Val	Val	
				225					230					235		
TGT	TCC	CTG	GAC	GGG	CTG	TTC	CCA	GTC	TCG	GAG	GCC	CAG	GTC	CAC	CTG	768
Cys	Ser	Leu	Asp	Gly	Leu	Phe	Pro	Val	Ser	Glu	Ala	Gln	Val	His	Leu	
			240					245					250			
GCA	CTG	GGG	GAC	CAG	AGG	TTG	AAC	CCC	ACA	GTC	ACC	TAT	GGC	AAC	GAC	816
Ala	Leu	Gly	Asp	Gln	Arg	Leu	Asn	Pro	Thr	Val	Thr	Tyr	Gly	Asn	Asp	
		255					260					265				
TCC	TTC	TCG	GCC	AAG	GCC	TCA	GTC	AGT	GTG	ACC	GCA	GAG	GAC	GAG	GGC	864
Ser	Phe	Ser	Ala	Lys	Ala	Ser	Val	Ser	Val	Thr	Ala	Glu	Asp	Glu	Gly	
	270					275					280					
ACC	CAG	CGG	CTG	ACG	TGT	GCA	GTA	ATA	CTG	GGG	AAC	CAG	AGC	CAG	GAG	912
Thr	Gln	Arg	Leu	Thr	Cys	Ala	Val	Ile	Leu	Gly	Asn	Gln	Ser	Gln	Glu	
	285				290					295					300	
ACA	CTG	CAG	ACA	GTG	ACC	ATC	TAC	AGC	TTT	CCG	GCG	CCC	AAC	GTG	ATT	960
Thr	Leu	Gln	Thr	Val	Thr	Ile	Tyr	Ser	Phe	Pro	Ala	Pro	Asn	Val	Ile	
				305					310					315		
CTG	ACG	AAG	CCA	GAG	GTC	TCA	GAA	GGG	ACC	GAG	GTG	ACA	GTG	AAG	TGT	1008
Leu	Thr	Lys	Pro	Glu	Val	Ser	Glu	Gly	Thr	Glu	Val	Thr	Val	Lys	Cys	
			320					325					330			
GAG	GCC	CAC	CCT	AGA	GCC	AAG	GTG	ACG	CTG	AAT	GGG	GTT	CCA	GCC	CAG	1056
Glu	Ala	His	Pro	Arg	Ala	Lys	Val	Thr	Leu	Asn	Gly	Val	Pro	Ala	Gln	
		335					340					345				
CCA	CTG	GGC	CCG	AGG	GCC	CAG	CTC	CTG	CTG	AAG	GCC	ACC	CCA	GAG	GAC	1104
Pro	Leu	Gly	Pro	Arg	Ala	Gln	Leu	Leu	Leu	Lys	Ala	Thr	Pro	Glu	Asp	
			350			355					360					
AAC	GGG	CGC	AGC	TTC	TCC	TGC	TCT	GCA	ACC	CTG	GAG	GTG	GCC	GGC	CAG	1152
Asn	Gly	Arg	Ser	Phe	Ser	Cys	Ser	Ala	Thr	Leu	Glu	Val	Ala	Gly	Gln	
	365				370					375					380	
CTT	ATA	CAC	AAG	AAC	CAG	ACC	CGG	GAG	CTT	CGT	GTC	CTG	TAT	GGC	CCC	1200
Leu	Ile	His	Lys	Asn	Gln	Thr	Arg	Glu	Leu	Arg	Val	Leu	Tyr	Gly	Pro	
				385					390					395		
CGA	CTG	GAC	GAG	AGG	GAT	TGT	CCG	GGA	AAC	TGG	ACG	TGG	CCA	GAA	AAT	1248
Arg	Leu	Asp	Glu	Arg	Asp	Cys	Pro	Gly	Asn	Trp	Thr	Trp	Pro	Glu	Asn	
			400					405					410			

54

TCC CAG CAG ACT CCA ATG TGC CAG GCT TGG GGG AAC CCA TTG CCC GAG	1296
Ser Gln Gln Thr Pro Met Cys Gln Ala Trp Gly Asn Pro Leu Pro Glu	
415 420 425	
CTC AAG TGT CTA AAG GAT GGC ACT TTC CCA CTG CCC ATC GGG GAA TCA	1344
Leu Lys Cys Leu Lys Asp Gly Thr Phe Pro Leu Pro Ile Gly Glu Ser	
430 435 440	
GTG ACT GTC ACT CGA GAT CTT GAG GGC ACC TAC CTC TGT CGG GCC AGG	1392
Val Thr Val Thr Arg Asp Leu Glu Gly Thr Tyr Leu Cys Arg Ala Arg	
445 450 455 460	
AGC ACT CAA GGG GAG GTC ACC CGC GAG GTG ACC GTG AAT GTG CTC TCC	1440
Ser Thr Gln Gly Glu Val Thr Arg Glu Val Thr Val Asn Val Leu Ser	
465 470 475	
CCC CGG TAT GAG ATT GTC ATC ATC ACT GTG GTA GCA GCC GCA GTC ATA	1488
Pro Arg Tyr Glu Ile Val Ile Ile Thr Val Val Ala Ala Ala Val Ile	
480 485 490	
ATG GGC ACT GCA GGC CTC AGC ACG TAC CTC TAT AAC CGC CAG CGG AAG	1536
Met Gly Thr Ala Gly Leu Ser Thr Tyr Leu Tyr Asn Arg Gln Arg Lys	
495 500 505	
ATC AAG AAA TAC AGA CTA CAA CAG GCC CAA AAA GGG ACC CCC ATG AAA	1584
Ile Lys Lys Tyr Arg Leu Gln Gln Ala Gln Lys Gly Thr Pro Met Lys	
510 515 520	
CCG AAC ACA CAA GCC ACG CCT CCC TGAACCTATC CCGGGACAGG GCCTCTTCCT	1638
Pro Asn Thr Gln Ala Thr Pro Pro	
525 530	
CGGCCTTCCC ATATTGGTGG CAGTGGTGCC ACACTGAACA GAGTGAAGA CATATGCCAT	1698
GCAGCTACAC CTACCGGCCC TGGGACGCCG GAGGACAGGG CATTGTCCTC AGTCAGATAC	1758
AACAGCATTG GGGGCCATGG TACCTGCACA CCTAAAACAC TAGGCCACGC ATCTGATCTG	1818
TAGTCACATG ACTAAGCCAA GAGGAAGG	1846

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 532 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Ala Pro Ser Ser Pro Arg Pro Ala Leu Pro Ala Leu Leu Val Leu
 1 5 10 15

Leu Gly Ala Leu Phe Pro Gly Pro Gly Asn Ala Gln Thr Ser Val Ser
 20 25 30

Pro Ser Lys Val Ile Leu Pro Arg Gly Gly Ser Val Leu Val Thr Cys
 35 40 45

Ser Thr Ser Cys Asp Gln Pro Lys Leu Leu Gly Ile Glu Thr Pro Leu
 50 55 60

Pro Lys Lys Glu Leu Leu Pro Gly Asn Asn Arg Lys Val Tyr Glu
 65 70 75 80

55

Leu Ser Asn Val Gln Glu Asp Ser Gln Pro Met Cys Tyr Ser Asn Cys
 85 90 95
 Pro Asp Gly Gln Ser Thr Ala Lys Thr Phe Leu Thr Val Tyr Trp Thr
 100 105 110
 Pro Glu Arg Val Glu Leu Ala Pro Leu Pro Ser Trp Gln Pro Val Gly
 115 120 125
 Lys Asn Leu Thr Leu Arg Cys Gln Val Glu Gly Gly Ala Pro Arg Ala
 130 135 140
 Asn Leu Thr Val Val Leu Leu Arg Gly Glu Lys Glu Leu Lys Arg Glu
 145 150 155 160
 Pro Ala Val Gly Glu Pro Ala Glu Val Thr Thr Thr Val Leu Val Arg
 165 170 175
 Arg Asp His His Gly Ala Asn Phe Ser Cys Arg Thr Glu Leu Asp Leu
 180 185 190
 Arg Pro Gln Gly Leu Glu Leu Phe Glu Asn Thr Ser Ala Pro Tyr Gln
 195 200 205
 Leu Gln Thr Phe Val Leu Pro Ala Thr Pro Pro Gln Leu Val Ser Pro
 210 215 220
 Arg Val Leu Glu Val Asp Thr Gln Gly Thr Val Val Cys Ser Leu Asp
 225 230 235 240
 Gly Leu Phe Pro Val Ser Glu Ala Gln Val His Leu Ala Leu Gly Asp
 245 250 255
 Gln Arg Leu Asn Pro Thr Val Thr Tyr Gly Asn Asp Ser Phe Ser Ala
 260 265 270
 Lys Ala Ser Val Ser Val Thr Ala Glu Asp Glu Gly Thr Gln Arg Leu
 275 280 285
 Thr Cys Ala Val Ile Leu Gly Asn Gln Ser Gln Glu Thr Leu Gln Thr
 290 295 300
 Val Thr Ile Tyr Ser Phe Pro Ala Pro Asn Val Ile Leu Thr Lys Pro
 305 310 315 320
 Glu Val Ser Glu Gly Thr Glu Val Thr Val Lys Cys Glu Ala His Pro
 325 330 335
 Arg Ala Lys Val Thr Leu Asn Gly Val Pro Ala Gln Pro Leu Gly Pro
 340 345 350
 Arg Ala Gln Leu Leu Leu Lys Ala Thr Pro Glu Asp Asn Gly Arg Ser
 355 360 365
 Phe Ser Cys Ser Ala Thr Leu Glu Val Ala Gly Gln Leu Ile His Lys
 370 375 380
 Asn Gln Thr Arg Glu Leu Arg Val Leu Tyr Gly Pro Arg Leu Asp Glu
 385 390 395 400
 Arg Asp Cys Pro Gly Asn Trp Thr Trp Pro Glu Asn Ser Gln Gln Thr
 405 410 415
 Pro Met Cys Gln Ala Trp Gly Asn Pro Leu Pro Glu Leu Lys Cys Leu
 420 425 430

56

Lys Asp Gly Thr Phe Pro Leu Pro Ile Gly Glu Ser Val Thr Val Thr
 435 440 445
 Arg Asp Leu Glu Gly Thr Tyr Leu Cys Arg Ala Arg Ser Thr Gln Gly
 450 455 460
 Glu Val Thr Arg Glu Val Thr Val Asn Val Leu Ser Pro Arg Tyr Glu
 465 470 475 480
 Ile Val Ile Ile Thr Val Val Ala Ala Ala Val Ile Met Gly Thr Ala
 485 490 495
 Gly Leu Ser Thr Tyr Leu Tyr Asn Arg Gln Arg Lys Ile Lys Lys Tyr
 500 505 510
 Arg Leu Gln Gln Ala Gln Lys Gly Thr Pro Met Lys Pro Asn Thr Gln
 515 520 525
 Ala Thr Pro Pro
 530

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2522 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 23..1636
- (D) OTHER INFORMATION: /product= "mouse intercellular adhesion molecule 1 (ICAM-1)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AGCACTCTGC CCTGGCCCTG CA ATG GCT TCA ACC CGT GCC AAG CCC ACG CTA	52
Met Ala Ser Thr Arg Ala Lys Pro Thr Leu	
1 5 10	
CCT CTG CTC CTG GCC CTG GTC ACC GTT GTG ATC CCT GGG CCT GGT GAT	100
Pro Leu Leu Leu Ala Leu Val Thr Val Val Ile Pro Gly Pro Gly Asp	
15 20 25	
GCT CAG GTA TCC ATC CAT CCC AGA GAA GCC TTC CTG CCC CAG GGT GGG	148
Ala Gln Val Ser Ile His Pro Arg Glu Ala Phe Leu Pro Gln Gly Gly	
30 35 40	
TCC GTG CAG GTG AAC TGT TCT TCC TCA TGC AAG GAG GAC CTC AGC CTG	196
Ser Val Gln Val Asn Cys Ser Ser Ser Cys Lys Glu Asp Leu Ser Leu	
45 50 55	
GGC TTG GAG ACT CAG TGG CTG AAA GAT GAG CTC GAG AGT GGA CCC AAC	244
Gly Leu Glu Thr Gln Trp Leu Lys Asp Glu Leu Glu Ser Gly Pro Asn	
60 65 70	
TGG AAG CTG TTT GAG CTG AGC GAG ATC GGG GAG GAC AGC AGT CCG CTG	292
Trp Lys Leu Phe Glu Leu Ser Glu Ile Gly Glu Asp Ser Ser Pro Leu	
75 80 85 90	

57

TGC	TTT	GAG	AAC	TGT	GGC	ACC	GTG	CAG	TCG	TCC	GCT	TCC	GCT	ACC	ATC	340
Cys	Phe	Glu	Asn	Cys	Gly	Thr	Val	Gln	Ser	Ser	Ala	Ser	Ala	Thr	Ile	
			95						100					105		
ACC	GTG	TAT	TCG	TTT	CCG	GAG	AGT	GTG	GAG	CTG	AGA	CCT	CTA	CCA	GCC	388
Thr	Val	Tyr	Ser	Phe	Pro	Glu	Ser	Val	Glu	Leu	Arg	Pro	Leu	Pro	Ala	
			110					115					120			
TGG	CAG	CAA	GTA	GGC	AAG	GAC	CTC	ACC	CTG	CGC	TGC	CAC	GTG	GAT	GGT	436
Trp	Gln	Gln	Val	Gly	Lys	Asp	Leu	Thr	Leu	Arg	Cys	His	Val	Asp	Gly	
		125					130					135				
GGA	GCA	CCG	CGG	ACC	CAG	CTC	TCA	GCA	GTG	CTG	CTC	CGT	GGG	GAG	GAG	484
Gly	Ala	Pro	Arg	Thr	Gln	Leu	Ser	Ala	Val	Leu	Leu	Arg	Gly	Glu	Glu	
	140					145					150					
ATA	CTG	AGC	CGC	CAG	CCA	GTG	GGT	GGG	CAC	CCC	AAG	GAC	CCC	AAG	GAG	532
Ile	Leu	Ser	Arg	Gln	Pro	Val	Gly	Gly	His	Pro	Lys	Asp	Pro	Lys	Glu	
	155				160					165					170	
ATC	ACA	TTC	ACG	GTG	CTG	GCT	AGC	AGA	GGG	GAC	CAC	GGA	GCC	AAT	TTC	580
Ile	Thr	Phe	Thr	Val	Leu	Ala	Ser	Arg	Gly	Asp	His	Gly	Ala	Asn	Phe	
			175						180					185		
TCA	TGC	CGC	ACA	GAA	CTG	GAT	CTC	AGG	CCG	CAA	GGG	CTG	GCA	TTG	TTC	628
Ser	Cys	Arg	Thr	Glu	Leu	Asp	Leu	Arg	Pro	Gln	Gly	Leu	Ala	Leu	Phe	
			190					195					200			
TCT	AAT	GTC	TCC	GAG	GCC	AGG	AGC	CTC	CGG	ACT	TTC	GAT	CTT	CCA	GCT	676
Ser	Asn	Val	Ser	Glu	Ala	Arg	Ser	Leu	Arg	Thr	Phe	Asp	Leu	Pro	Ala	
		205					210					215				
ACC	ATC	CCA	AAG	CTC	GAC	ACC	CCT	GAC	CTC	CTG	GAG	GTG	GGC	ACC	CAG	724
Thr	Ile	Pro	Lys	Leu	Asp	Thr	Pro	Asp	Leu	Leu	Glu	Val	Gly	Thr	Gln	
	220					225					230					
CAG	AAG	TTG	TTT	TGC	TCC	CTG	GAA	GGC	CTG	TTT	CCT	GCC	TCT	GAA	GCT	772
Gln	Lys	Leu	Phe	Cys	Ser	Leu	Glu	Gly	Leu	Phe	Pro	Ala	Ser	Glu	Ala	
	235				240				245						250	
CGG	ATA	TAC	CTG	GAG	CTG	GGA	GGC	CAG	ATG	CCG	ACC	CAG	GAG	AGC	ACA	820
Arg	Ile	Tyr	Leu	Glu	Leu	Gly	Gly	Gln	Met	Pro	Thr	Gln	Glu	Ser	Thr	
			255						260					265		
AAC	AGC	AGT	GAC	TCT	GTG	TCA	GCC	ACT	GCC	TTG	GTA	GAG	GTG	ACT	GAG	868
Asn	Ser	Ser	Asp	Ser	Val	Ser	Ala	Thr	Ala	Leu	Val	Glu	Val	Thr	Glu	
			270					275					280			
GAG	TTC	GAC	AGA	ACC	CTG	CCG	CTG	CGC	TGC	GTT	TTG	GAG	CTA	GCG	GAC	916
Glu	Phe	Asp	Arg	Thr	Leu	Pro	Leu	Arg	Cys	Val	Leu	Glu	Leu	Ala	Asp	
		285					290					295				
CAG	ATC	CTG	GAG	ACG	CAG	AGG	ACC	TTA	ACA	GTC	TAC	AAC	TTT	TCA	GCT	964
Gln	Ile	Leu	Glu	Thr	Gln	Arg	Thr	Leu	Thr	Val	Tyr	Asn	Phe	Ser	Ala	
	300					305					310					
CCG	GTC	CTG	ACC	CTG	AGC	CAG	CTG	GAG	GTC	TCG	GAA	GGG	AGC	CAA	GTA	1012
Pro	Val	Leu	Thr	Leu	Ser	Gln	Leu	Glu	Val	Ser	Glu	Gly	Ser	Gln	Val	
	315				320					325					330	
ACT	GTG	AAG	TGT	GAA	GCC	CAC	AGT	GGG	TCG	AAG	GTG	GTT	CTT	CTG	AGC	1060
Thr	Val	Lys	Cys	Glu	Ala	His	Ser	Gly	Ser	Lys	Val	Val	Leu	Leu	Ser	
			335					340					345			
GGC	GTC	GAG	CCT	AGG	CCA	CCC	ACC	CCG	CAG	GTC	CAA	TTC	ACA	CTG	AAT	1108
Gly	Val	Glu	Pro	Arg	Pro	Pro	Thr	Pro	Gln	Val	Gln	Phe	Thr	Leu	Asn	
			350					355					360			

GCC AGC TCG GAG GAT CAC AAA CGA AGC TTC TTT TGC TCT GCC GCT CTG	1156
Ala Ser Ser Glu Asp His Lys Arg Ser Phe Phe Cys Ser Ala Ala Leu	
365 370 375	
GAG GTG GCG GGA AAG TTC CTG TTT AAA AAC CAG ACC CTG GAA CTG CAC	1204
Glu Val Ala Gly Lys Phe Leu Phe Lys Asn Gln Thr Leu Glu Leu His	
380 385 390	
GTG CTG TAT GGT CCT CGG CTG GAC GAG ACG GAC TGC TTG GGG AAC TGG	1252
Val Leu Tyr Gly Pro Arg Leu Asp Glu Thr Asp Cys Leu Gly Asn Trp	
395 400 405 410	
ACC TGG CAA GAG GGG TCT CAG CAG ACT CTG AAA TGC CAG GCC TGG GGG	1300
Thr Trp Gln Glu Ser Gln Gln Thr Leu Lys Cys Gln Ala Trp Gly	
415 420 425	
AAC CCA TCT CCT AAA ATG ACC TGC AGA CGG AAG GCA GAT GGT GCC CTG	1348
Asn Pro Ser Pro Lys Met Thr Cys Arg Arg Lys Ala Asp Gly Ala Leu	
430 435 440	
CTG CCC ATC GGG GTG GTG AAG TCT GTC AAA CAG GAG ATG AAT GGT ACA	1396
Leu Pro Ile Gly Val Val Lys Ser Val Lys Gln Glu Met Asn Gly Thr	
445 450 455	
TAC GTG TGC CAT GCC TTT AGC TCC CAT GGG AAT GTC ACC AGG AAT GTG	1444
Tyr Val Cys His Ala Phe Ser Ser His Gly Asn Val Thr Arg Asn Val	
460 465 470	
TAC CTG ACA GTA CTG TAC CAC TCT CAA AAT AAC TGG ACT ATA ATC ATT	1492
Tyr Leu Thr Val Leu Tyr His Ser Gln Asn Asn Trp Thr Ile Ile Ile	
475 480 485 490	
CTG GTG CCA GTA CTG CTG GTC ATT GTG GGC CTC GTG ATG GCA GCC TCT	1540
Leu Val Pro Val Leu Leu Val Ile Val Gly Leu Val Met Ala Ala Ser	
495 500 505	
TAT GTT TAT AAC CGC CAG AGA AAG ATC AGG ATA TAC AAG TTA CAG AAG	1588
Tyr Val Tyr Asn Arg Gln Arg Lys Ile Arg Ile Tyr Lys Leu Gln Lys	
510 515 520	
GCT CAG GAG GAG GCC ATA AAA CTC AAG GGA CAA GCC CCA CCT CCC	1633
Ala Gln Glu Glu Ala Ile Lys Leu Lys Gly Gln Ala Pro Pro Pro	
525 530 535	
TGAGCCTGCT GGATGAGACT CCTGCCTGGA CCCCCTGCAG GGCAACAGCT GCTGCTGCTT	1693
TTGAACAGAA TGGTAGACAG CATTTACCCT CAGCCACTTC CTCTGGCTGT CACAGAACAG	1753
GATGGTGGCC TGGGGGATGC ACACTTGTAG CCTCAGAGCT AAGAGGACTC GGTGGATGGA	1813
GCAAGACTGT GAACACGTGT GACCCGGACC CACCTACAGC CCGGTGGACC TTCAGCCAAG	1873
AAACGCTGAC TTCATTCTCT ATTGCCCCCTG CTGAGGGGCT CCTGCCTAAG GAAGACATGA	1933
TATCCAGTAG ACACAAGCAA GAAGACCACA CTTCCCCCCC GACACAGGAA AGCTGAGACA	1993
TTGTCCCCAA CTCTTCTTGA TGTATTTATT AATTTAGAGT TTTACCAGCT ATTTATTGAG	2053
TACCCTGTAT ATAGTAGATC AGTGAGGAGG TGAATGTATA AGTTATGGCC TGGACCCTGC	2113
TGCAGATGCT GTGAGAGTCT GGGGAAAGAT CACATGGGTC GAGGGTTTCT CTA CTGGTCA	2173
GGATGCTTTT CTCATAAGGG TCGACTTTTT TCACCAGTCA CATAAACACT ATGTGGACTA	2233
GCAGTGGTTC TCTGCTCCTC CACATCCTGG AGCGTCCCAG CACCTCCCCA CCTACTTTTG	2293
TTCCCAATGT CAGCCACCAT GCCTTAGCAG CTGAACAATC GAGCCTCATG CTCATGAAAT	2353

CATGGTCCCA GCGGGCTCCA CCTCAAAGAG AAAGCCTGGA AGGAAATGTT CCAACTCCTT	2413
AGAAGGGTCG TGCAAGCTGC TGTGGGAGGG TAAGCACCCC TCCCAGCAGC AGAAACCTTT	2473
CCTTTGAATC AATAAAGTTT TATGTCGGCC TGAAAAAAAA AAAAAAAAAA	2522

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 537 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met	Ala	Ser	Thr	Arg	Ala	Lys	Pro	Thr	Leu	Pro	Leu	Leu	Leu	Ala	Leu	1	5	10	15
Val	Thr	Val	Val	Ile	Pro	Gly	Pro	Gly	Asp	Ala	Gln	Val	Ser	Ile	His	20	25	30	
Pro	Arg	Glu	Ala	Phe	Leu	Pro	Gln	Gly	Gly	Ser	Val	Gln	Val	Asn	Cys	35	40	45	
Ser	Ser	Ser	Cys	Lys	Glu	Asp	Leu	Ser	Leu	Gly	Leu	Glu	Thr	Gln	Trp	50	55	60	
Leu	Lys	Asp	Glu	Leu	Glu	Ser	Gly	Pro	Asn	Trp	Lys	Leu	Phe	Glu	Leu	65	70	75	80
Ser	Glu	Ile	Gly	Glu	Asp	Ser	Ser	Pro	Leu	Cys	Phe	Glu	Asn	Cys	Gly	85	90	95	
Thr	Val	Gln	Ser	Ser	Ala	Ser	Ala	Thr	Ile	Thr	Val	Tyr	Ser	Phe	Pro	100	105	110	
Glu	Ser	Val	Glu	Leu	Arg	Pro	Leu	Pro	Ala	Trp	Gln	Gln	Val	Gly	Lys	115	120	125	
Asp	Leu	Thr	Leu	Arg	Cys	His	Val	Asp	Gly	Gly	Ala	Pro	Arg	Thr	Gln	130	135	140	
Leu	Ser	Ala	Val	Leu	Leu	Arg	Gly	Glu	Glu	Ile	Leu	Ser	Arg	Gln	Pro	145	150	155	160
Val	Gly	Gly	His	Pro	Lys	Asp	Pro	Lys	Glu	Ile	Thr	Phe	Thr	Val	Leu	165	170	175	
Ala	Ser	Arg	Gly	Asp	His	Gly	Ala	Asn	Phe	Ser	Cys	Arg	Thr	Glu	Leu	180	185	190	
Asp	Leu	Arg	Pro	Gln	Gly	Leu	Ala	Leu	Phe	Ser	Asn	Val	Ser	Glu	Ala	195	200	205	
Arg	Ser	Leu	Arg	Thr	Phe	Asp	Leu	Pro	Ala	Thr	Ile	Pro	Lys	Leu	Asp	210	215	220	
Thr	Pro	Asp	Leu	Leu	Glu	Val	Gly	Thr	Gln	Gln	Lys	Leu	Phe	Cys	Ser	225	230	235	240
Leu	Glu	Gly	Leu	Phe	Pro	Ala	Ser	Glu	Ala	Arg	Ile	Tyr	Leu	Glu	Leu	245	250	255	

60

Gly	Gly	Gln		Met 260	Pro	Thr	Gln	Glu	Ser 265	Thr	Asn	Ser	Ser	Asp 270	Ser	Val
Ser	Ala	Thr 275	Ala	Leu	Val	Glu	Val 280	Thr	Glu	Glu	Phe	Asp 285	Arg	Thr	Leu	
Pro	Leu 290	Arg	Cys	Val	Leu	Glu 295	Leu	Ala	Asp	Gln	Ile 300	Leu	Glu	Thr	Gln	
Arg 305	Thr	Leu	Thr	Val	Tyr 310	Asn	Phe	Ser	Ala	Pro 315	Val	Leu	Thr	Leu	Ser 320	
Gln	Leu	Glu	Val	Ser 325	Glu	Gly	Ser	Gln	Val 330	Thr	Val	Lys	Cys	Glu 335	Ala	
His	Ser	Gly	Ser 340	Lys	Val	Val	Leu	Leu 345	Ser	Gly	Val	Glu	Pro 350	Arg	Pro	
Pro	Thr	Pro 355	Gln	Val	Gln	Phe	Thr 360	Leu	Asn	Ala	Ser	Ser 365	Glu	Asp	His	
Lys	Arg 370	Ser	Phe	Phe	Cys	Ser 375	Ala	Ala	Leu	Glu	Val 380	Ala	Gly	Lys	Phe	
Leu 385	Phe	Lys	Asn	Gln	Thr 390	Leu	Glu	Leu	His	Val 395	Leu	Tyr	Gly	Pro	Arg 400	
Leu	Asp	Glu	Thr	Asp 405	Cys	Leu	Gly	Asn	Trp 410	Thr	Trp	Gln	Glu	Gly 415	Ser	
Gln	Gln	Thr 420	Leu	Lys	Cys	Gln	Ala	Trp 425	Gly	Asn	Pro	Ser	Pro 430	Lys	Met	
Thr	Cys	Arg 435	Arg	Lys	Ala	Asp	Gly 440	Ala	Leu	Leu	Pro	Ile 445	Gly	Val	Val	
Lys	Ser 450	Val	Lys	Gln	Glu	Met 455	Asn	Gly	Thr	Tyr	Val 460	Cys	His	Ala	Phe	
Ser 465	Ser	His	Gly	Asn	Val 470	Thr	Arg	Asn	Val	Tyr 475	Leu	Thr	Val	Leu	Tyr 480	
His	Ser	Gln	Asn	Asn 485	Trp	Thr	Ile	Ile	Ile 490	Leu	Val	Pro	Val	Leu 495	Leu	
Val	Ile	Val 500	Gly	Leu	Val	Met	Ala	Ala 505	Ser	Tyr	Val	Tyr	Asn 510	Arg	Gln	
Arg	Lys	Ile 515	Arg	Ile	Tyr	Lys	Leu 520	Gln	Lys	Ala	Gln	Glu 525	Glu	Ala	Ile	
Lys	Leu 530	Lys	Gly	Gln	Ala	Pro 535	Pro	Pro								

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single.
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

61

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GCCCCAAGCTG GCATCCGTCA

20

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TGCATCCCCC AGGCCACCAT

20

WHAT IS CLAIMED IS:

1. A pharmaceutical composition for the treatment of pathologic conditions associated with the overexpression of ICAM-1 in a host, said pharmaceutical composition comprising an effective amount of an ICAM-1 antisense molecule encapsulated in a lipid mixture, said lipid mixture comprising at least two members selected from the group consisting of phospholipids, sterols and cationic lipids.
2. A pharmaceutical composition in accordance with claim 1, wherein said lipid mixture is in the form of liposomes and consists essentially of neutral phospholipids and a sterol.
3. A pharmaceutical composition in accordance with claim 1, wherein said lipid mixture is in the form of lipid particles.
4. A pharmaceutical composition in accordance with claim 1, wherein said ICAM-1 antisense molecule comprises from about 15 to about 50 nucleic acids and is complementary to a portion of the 3'-untranslated region of ICAM-1.
5. A pharmaceutical composition in accordance with claim 4, wherein said ICAM-1 antisense molecule is a phosphorothioate molecule.
6. A pharmaceutical composition in accordance with claim 4, wherein said ICAM-1 antisense molecule is a methyl phosphonate molecule.
7. A pharmaceutical composition in accordance with claim 1, wherein said lipid mixture is in the form of liposomes consisting essentially of phosphatidylcholine and a sterol.
8. A pharmaceutical composition in accordance with claim 1, wherein said lipid mixture is in the form of liposomes consisting essentially of egg phosphatidylcholine and cholesterol.

9. A pharmaceutical composition in accordance with claim 1, wherein said lipid mixture is in the form of liposomes consisting essentially of neutral phospholipids and cholesterol, said liposomes having diameters of from about 50 to about 150 nm.

10. A pharmaceutical composition in accordance with claim 9, wherein said liposomes have diameters of from about 75 to about 125 nm.

11. A pharmaceutical composition in accordance with claim 1, wherein said lipid mixture is in the form of lipid particles, said lipid particles being from about 50 to about 90 nm in diameter.

12. A pharmaceutical composition in accordance with claim 1, wherein said lipid mixture is in the form of lipid particles, said lipid particles being from about 60 to about 80 nm in diameter.

13. A pharmaceutical composition in accordance with claim 12, wherein said lipid particles comprise phospholipids and cationic lipids, wherein said cationic lipids are members selected from the group consisting of DODAC, DDAB, DOTAP, DOTMA, DOSPA, DOGS, DC-Chol and combinations thereof, and said phospholipids are members selected from the group consisting of DOPE, POPC, EPC and combinations thereof.

14. A method for the treatment of pathologic conditions associated with the overexpression of ICAM-1 in a host, said method comprising delivering to said host a pharmaceutical composition in accordance with claim 1.

15. A method in accordance with claim 14, wherein said composition is a liposomal composition.

16. A method in accordance with claim 14, wherein said composition comprises lipid particles.

17. A method in accordance with claim 14, wherein said ICAM-1 antisense molecule comprises from about 15 to about 50 nucleic acids and is complementary to a portion of the 3'-untranslated region of ICAM-1.

18. A method in accordance with claim 14, wherein said delivering comprises administering intravenously.

19. A method in accordance with claim 14, wherein said delivering comprises administering topically.

20. A method in accordance with claim 16, wherein said lipid particles comprise phospholipids and cationic lipids, said cationic lipids being members selected from the group consisting of DODAC, DDAB, DOTAP, DOTMA, DOSPA, DOGS, DC-Chol and combinations thereof, and said phospholipids being members selected from the group consisting of DOPE, POPC, EPC and combinations thereof.

21. A method in accordance with claim 14, wherein said condition is a member selected from the group consisting of Alzheimer's disease, multiple sclerosis, uveitis, Herpes keratitis, renal allograft rejection, glomerulonephritis, liver allograft rejection, viral hepatitis, alcoholic hepatitis, cholangitis, cardiac allograft rejection, atherosclerotic plaques, rheumatoid arthritis, Grave's disease, Hashimoto's thyroiditis, psoriasis, scleroderma, graft v host disease, contact dermatitis, lichen planus, fixed drug eruption, mycosis fungoides, and alopecia areata.

22. A method in accordance with claim 14, wherein said condition is contact dermatitis.

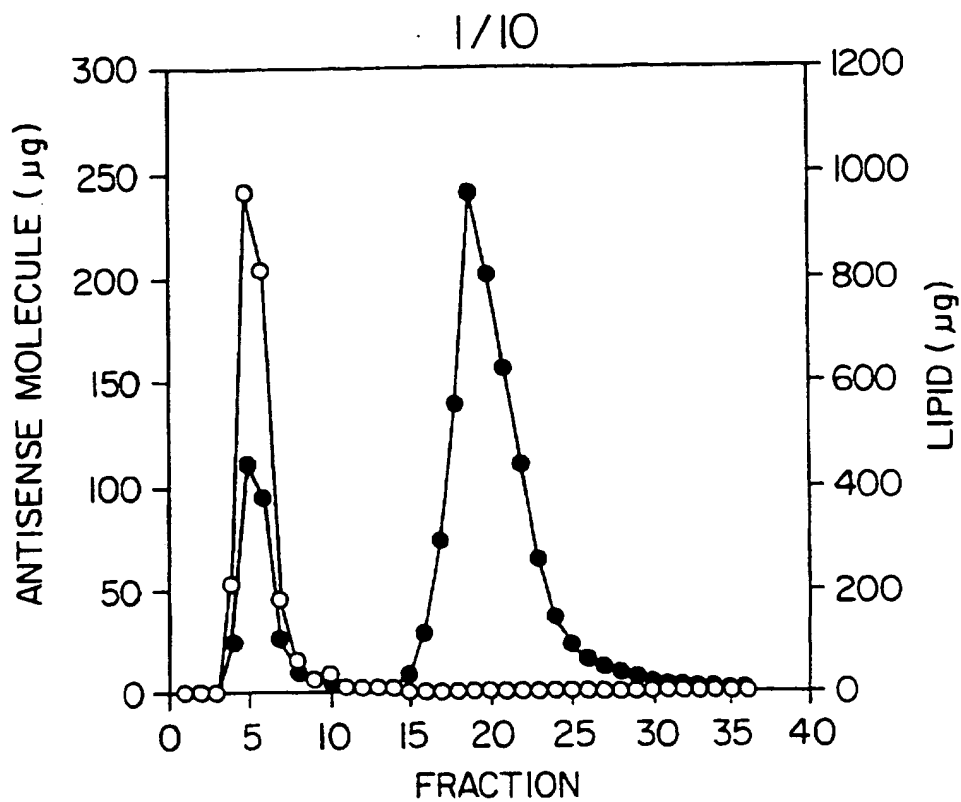


FIG. 1

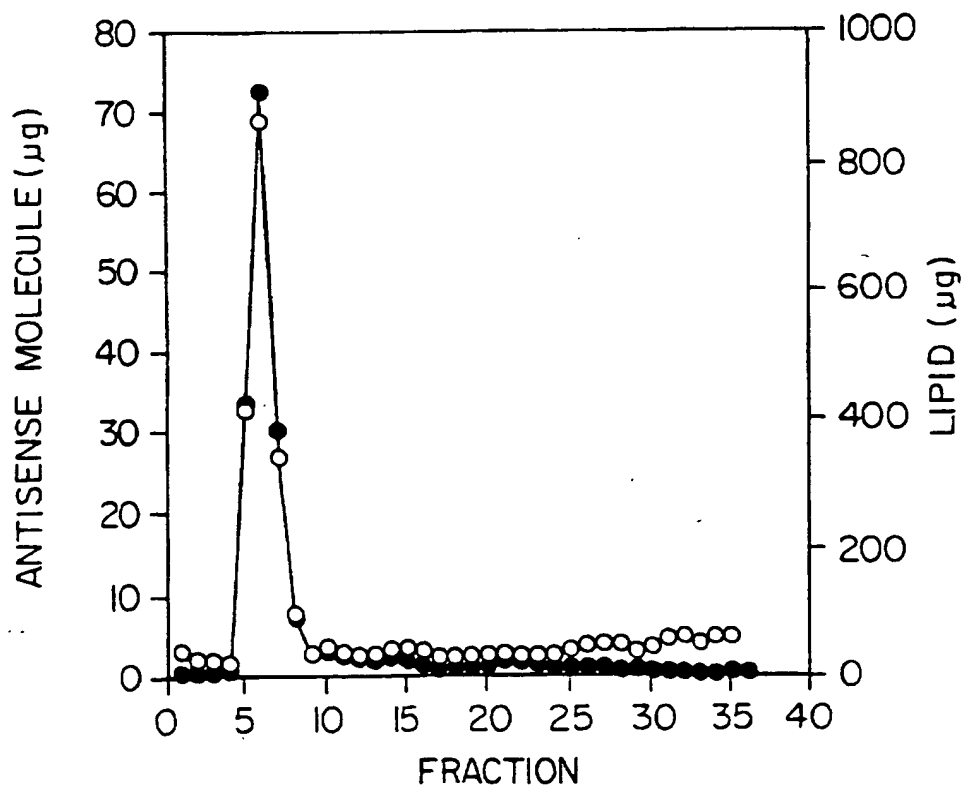


FIG. 2

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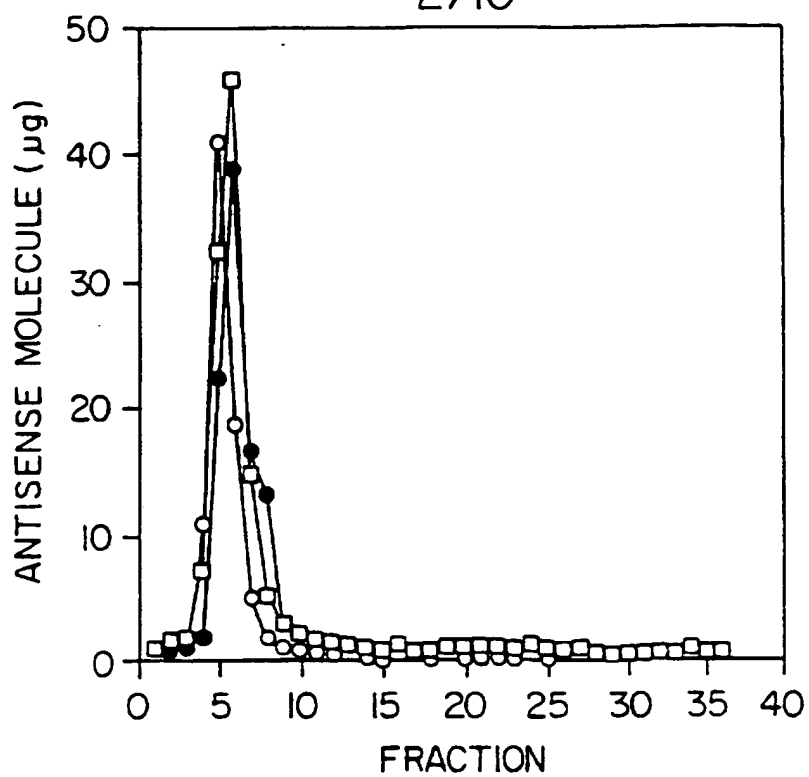


FIG. 3

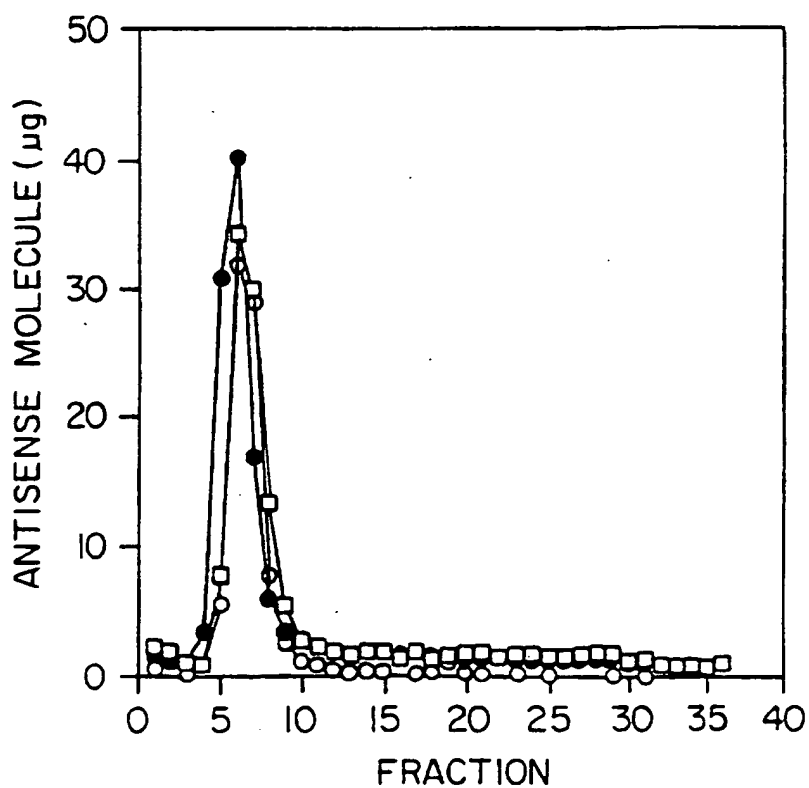


FIG. 4

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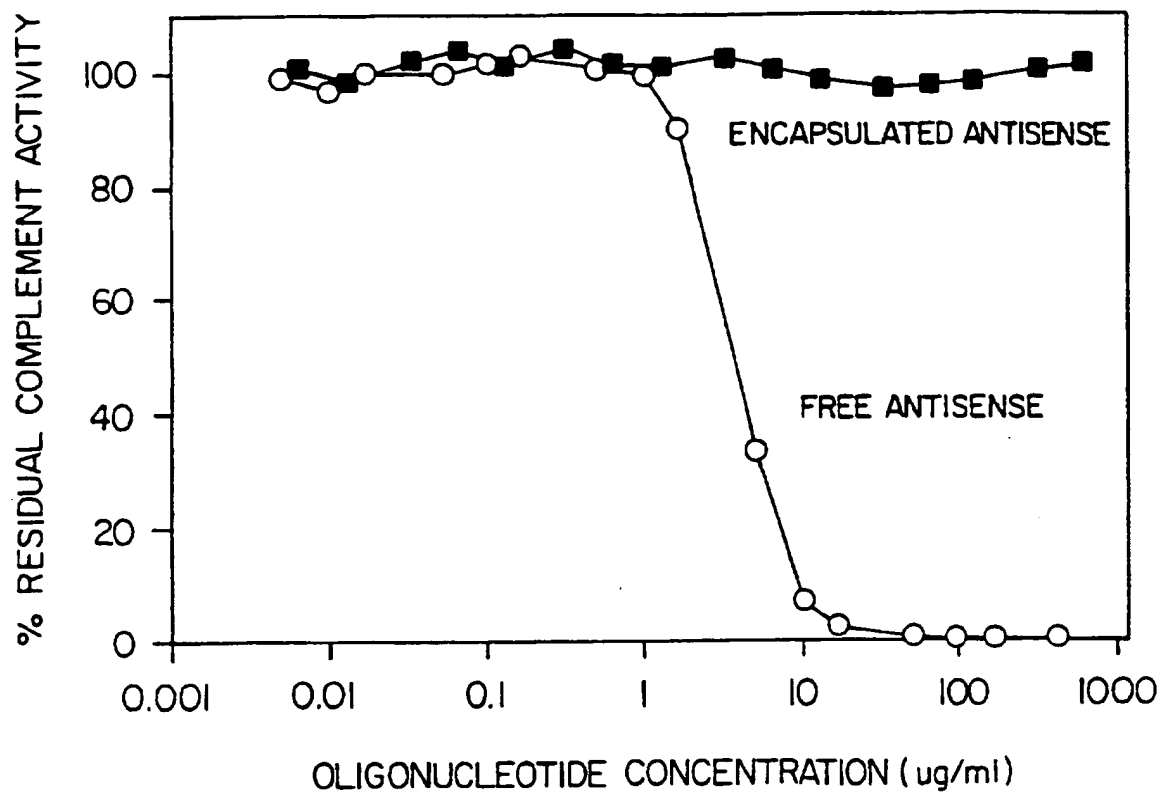
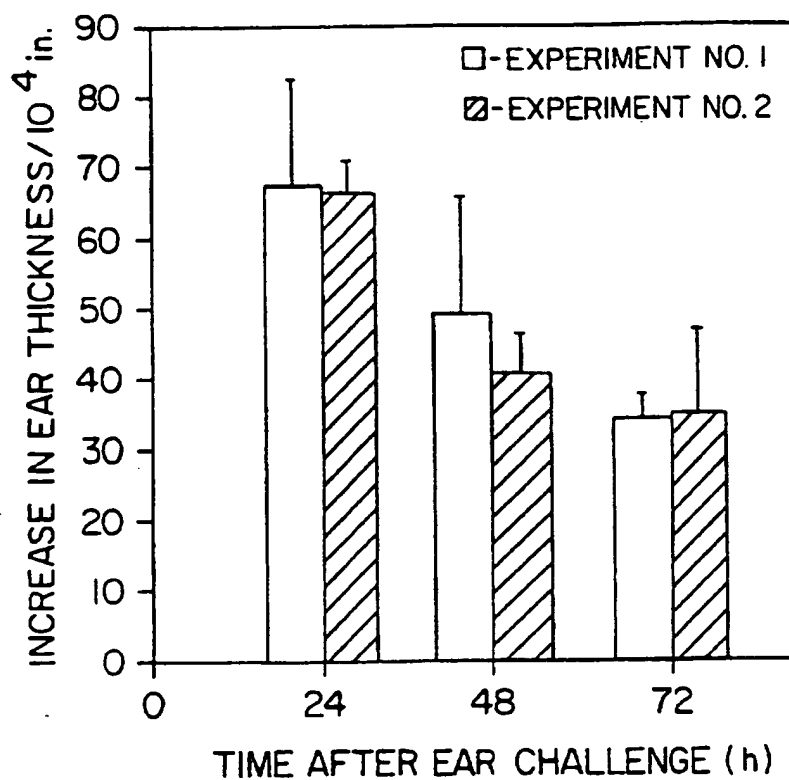
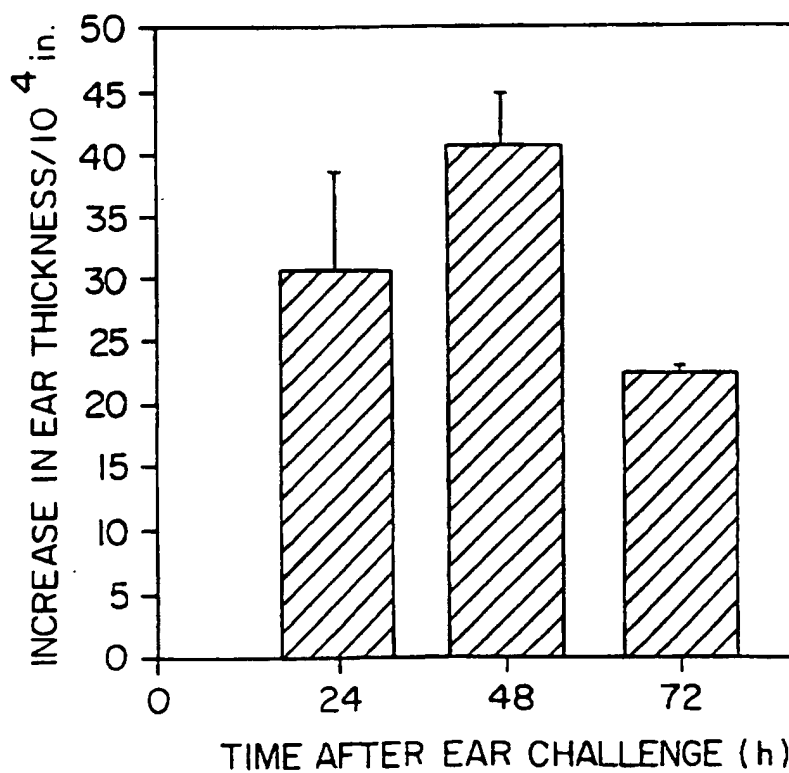


FIG. 5

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**FIG. 6****FIG. 7**

SUBSTITUTE SHEET (RULE 26)

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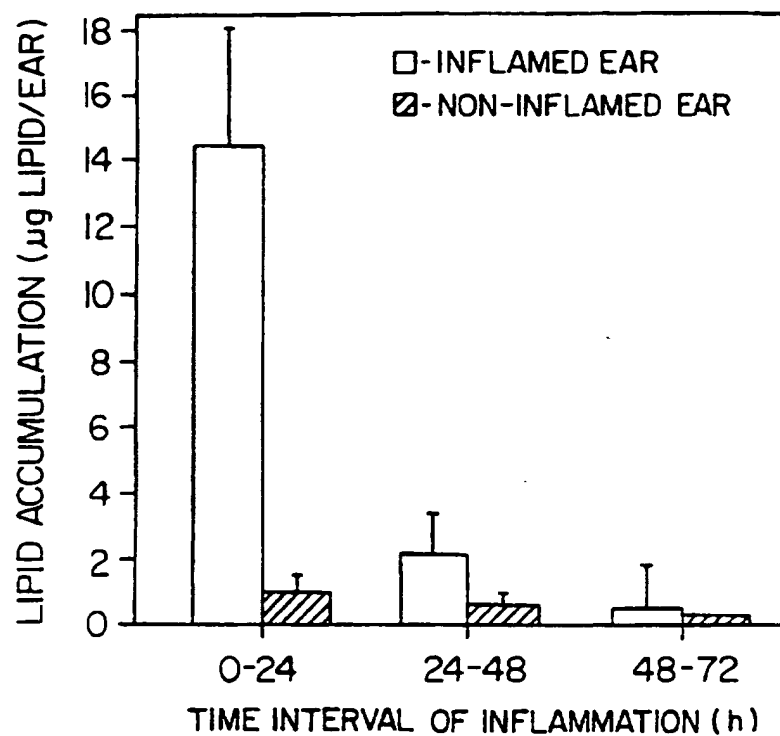


FIG. 8

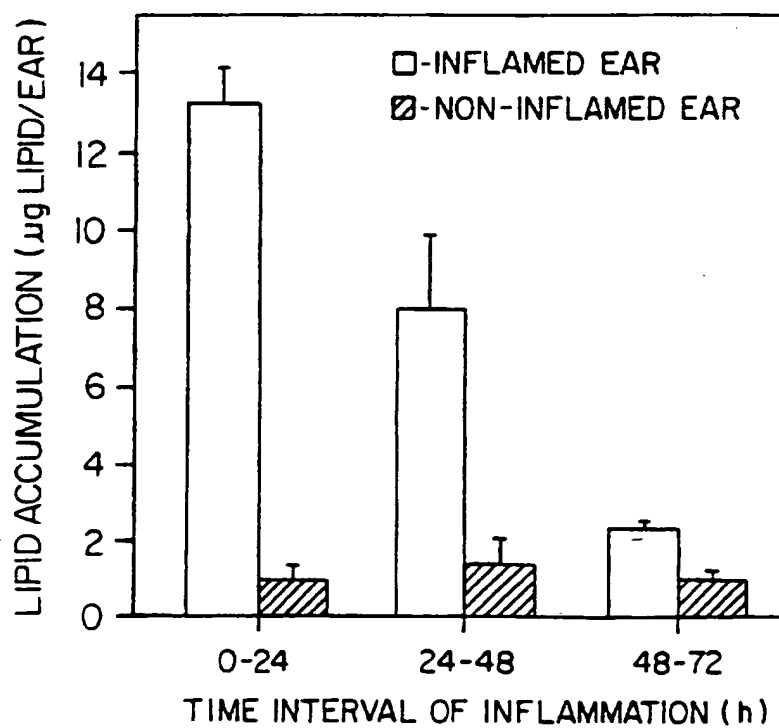


FIG. 9

SUBSTITUTE SHEET (RULE 26)

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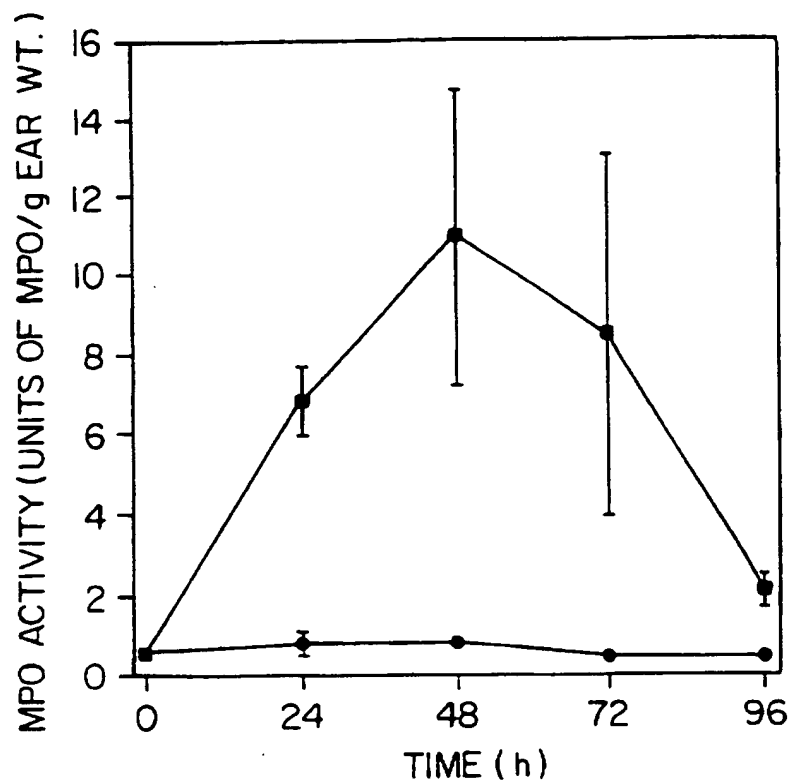


FIG. 10

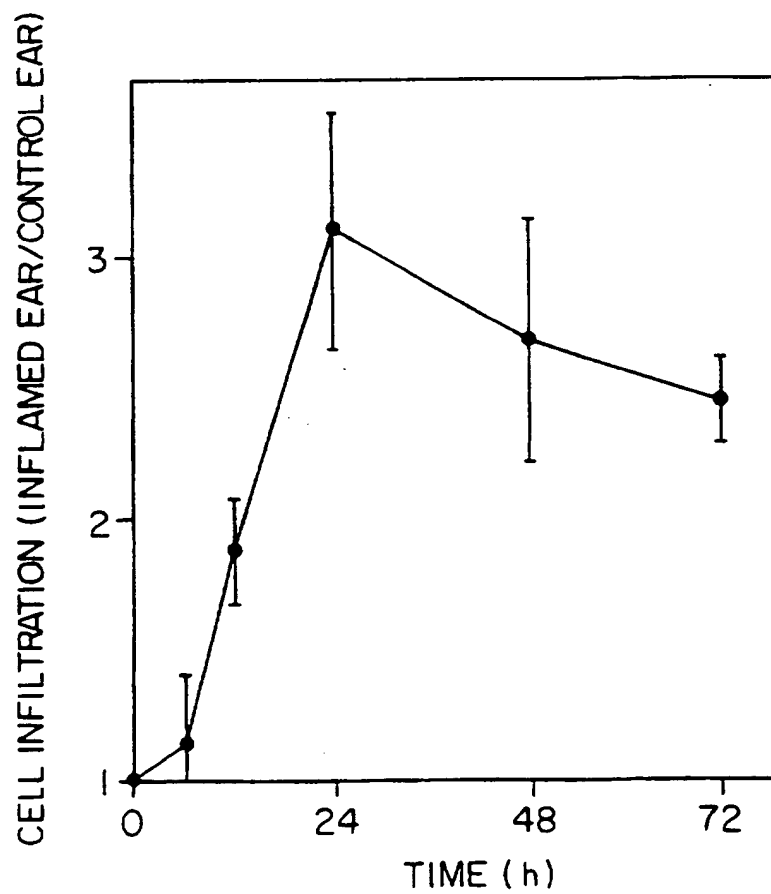


FIG. 11

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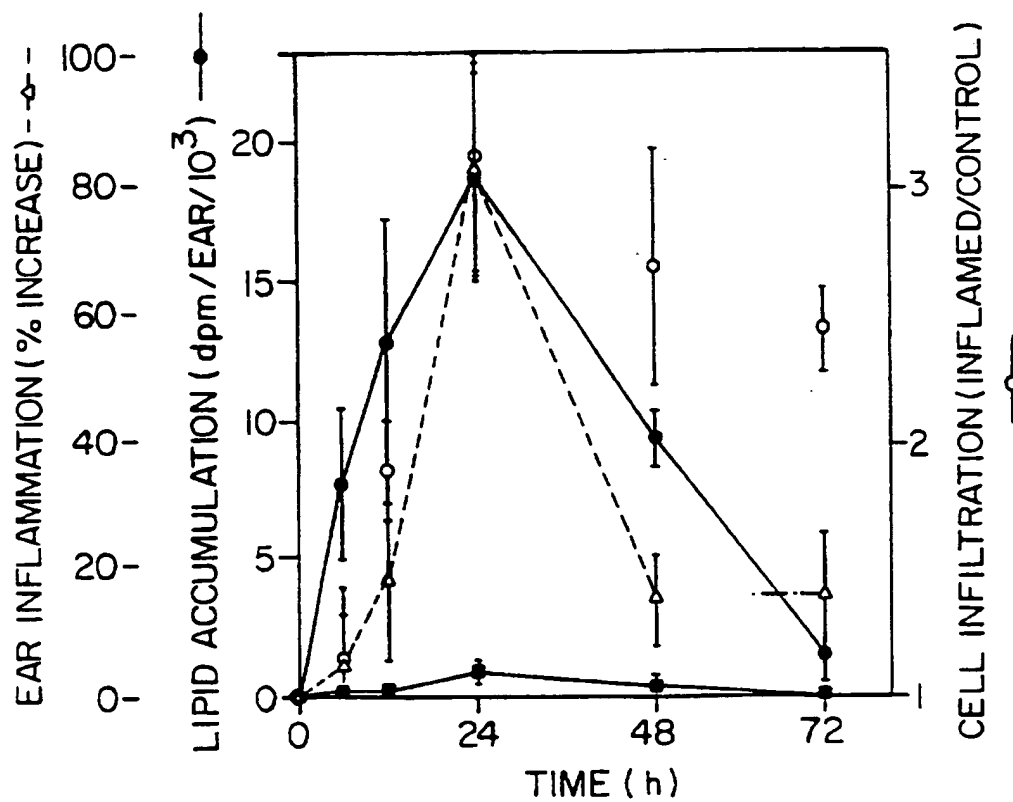


FIG. 12

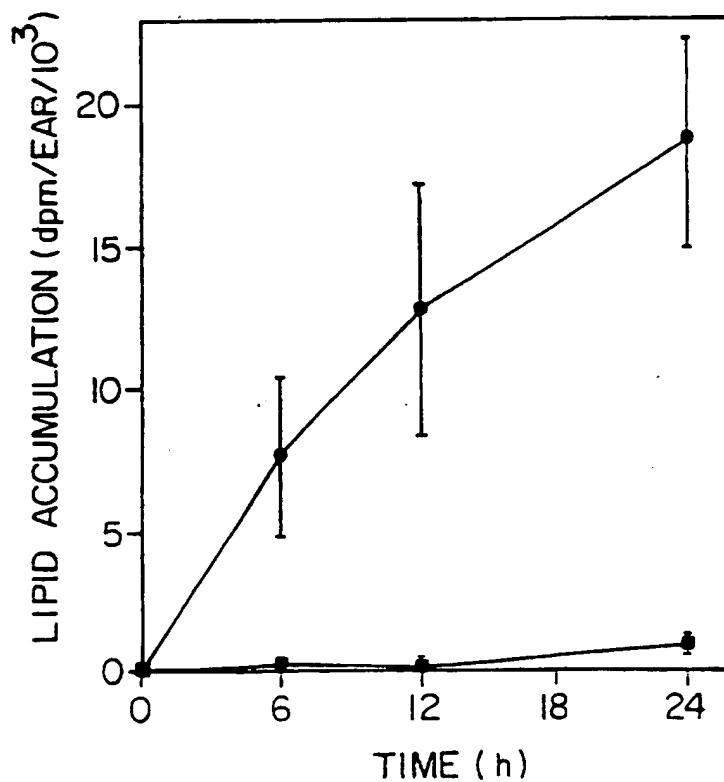


FIG. 13

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FIG. 14

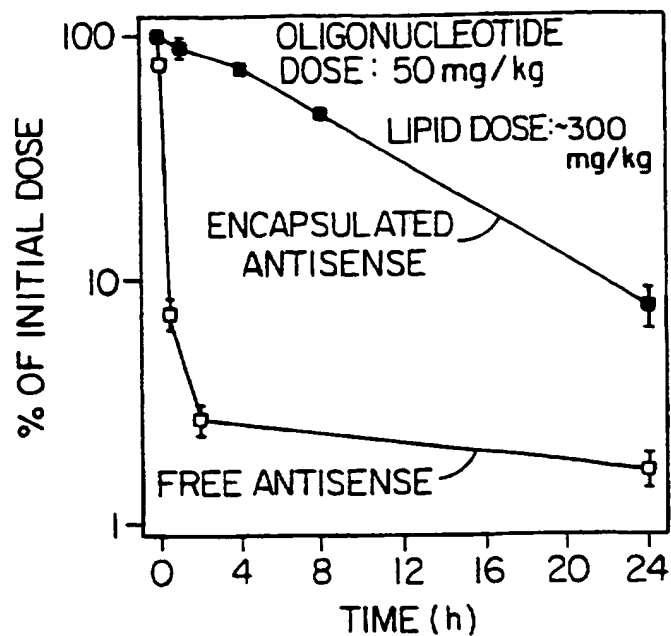
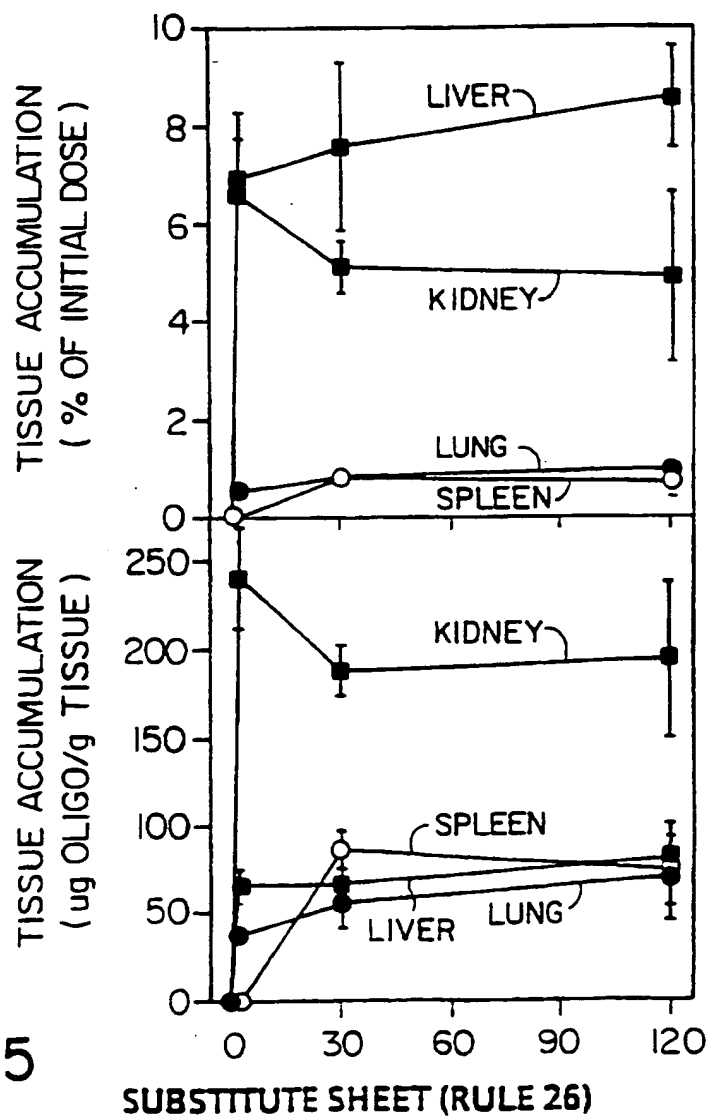


FIG. 15



SUBSTITUTE SHEET (RULE 26)

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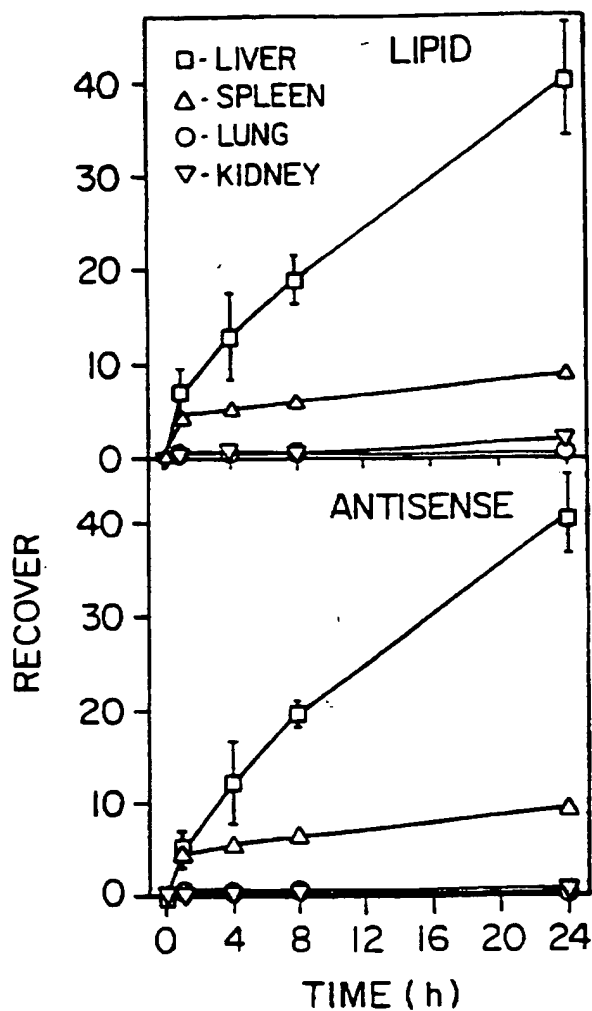


FIG. 16

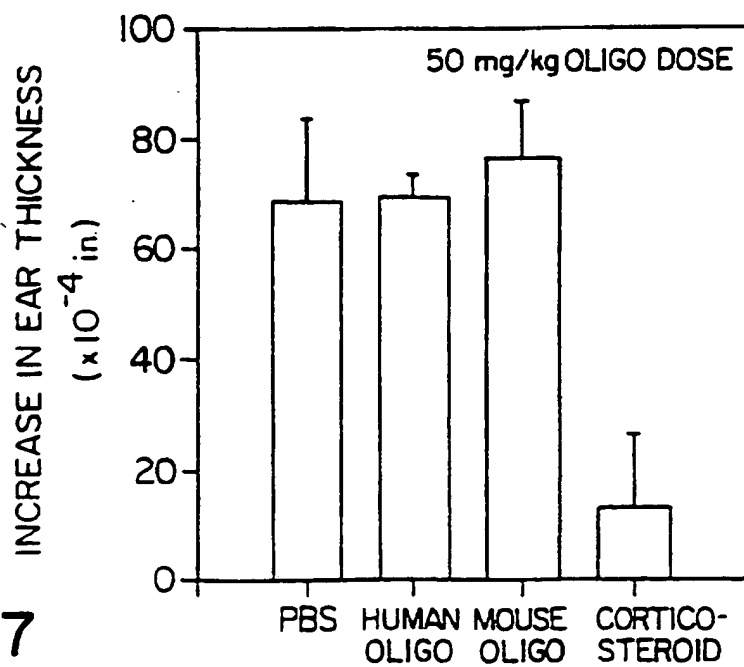


FIG. 17

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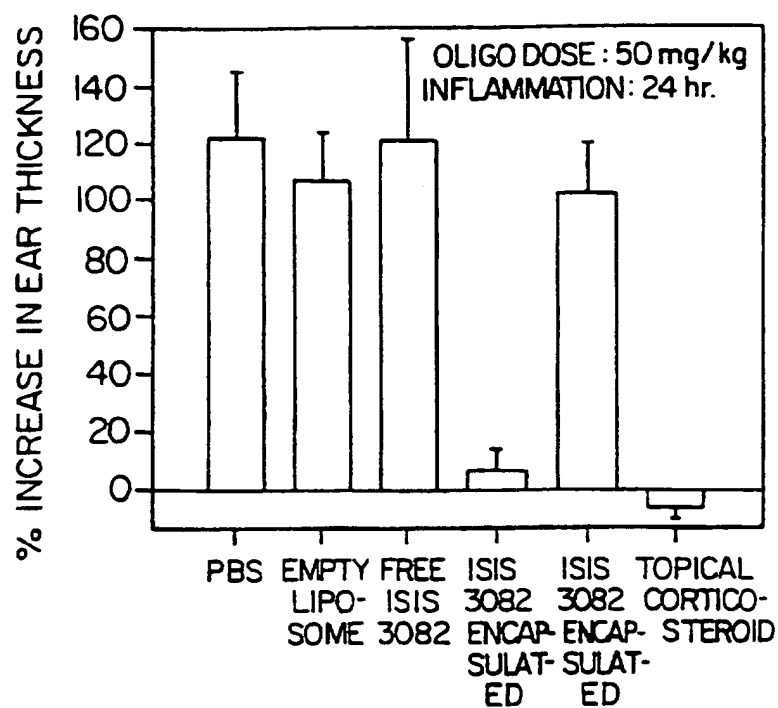


FIG. 18

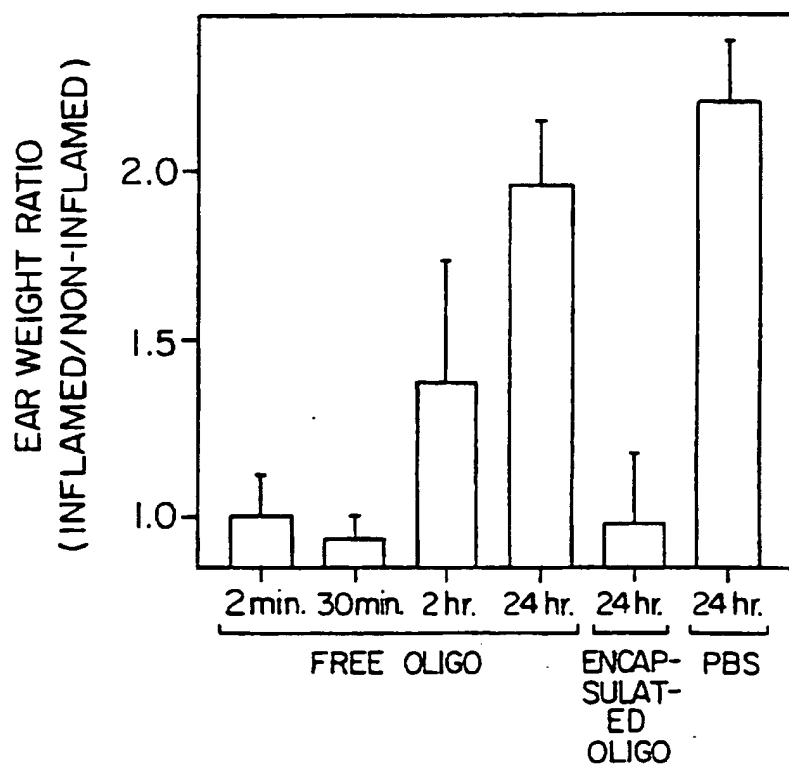


FIG. 19

INTERNATIONAL SEARCH REPORT

Internat'l Application No

PCT/CA 97/00347

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C12N15/11 A61K9/127 A61K31/70 C07H21/00 //C12N15/88

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 05333 A (ISIS PHARMACEUTICALS INC) 17 March 1994 cited in the application see page 4, line 10 - page 5, line 8 see page 11, line 28 - page 14, line 17 see page 26 - page 30, line 4	1,3-6, 14,16-22
Y	see examples 15-22	1-5, 7-17,19, 20
Y	--- WO 96 10390 A (INEX PHARMACEUTICALS CORP) 11 April 1996 cited in the application see page 9, line 14 - line 30 see page 12, line 20 - line 28 see examples 3-9 --- -/-	1,3-5, 11-14, 16,17,20

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

10 September 1997

Date of mailing of the international search report

30.09.97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
 Fax: (+31-70) 340-3016

Authorized officer

Andres, S

INTERNATIONAL SEARCH REPORT

Intern Application No

PCT/CA 97/00347

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 94 22468 A (ANTICANCER INC ; LI LINGNA (US); LISHKO VALERYI K (US)) 13 October 1994 see page 21 - page 25, line 17 see page 36, line 2 - page 37, line 1 see page 45, line 27 - page 46, line 31 see claims	1-3, 7-17, 19, 20
X	--- JOURNAL OF IMMUNOLOGY, vol. 153, 1994, BALTIMORE US, pages 5336-5346, XP002040367 STEPKOWSKI, S. ET AL.: "Blocking of heart allograft rejection by intercellular adhesion molecule-1 antisense oligonucleotides alone or in combination with other immunosuppressive modalities" see page 5337, right-hand column, paragraph 2-3 see page 5338, 'Results'	1,3-5, 14, 16-18, 20,21
A	--- JOURNAL OF CONTROLLED RELEASE, vol. 22, no. 1, 1 September 1992, pages 47-56, XP000294386 SAGHIR, A. & JULIANO, R.: "LIPOSOME DELIVERY OF ANTISENSE OLIGONUCLEOTIDES: ADSORPTION AND EFFLUX CHARACTERISTICS OF PHOSPHOROTHIOATE OLIGODEOXYNUCLEOTIDES" see the whole document	2,7,8,15
A	--- ERICKSON, R. & IZANT, J. 'GENE REGULATION: BIOLOGY OF ANTISENSE RNA AND DNA'; RAVEN PRESS, NEW YORK, US; 1992, pages 147-161, XP002040368 THIERRY, A. ET AL.: "Liposomal delivery as a new approach to transport antisense oligonucleotides"	
A	--- BURI, P. ET AL. 'FORMES PHARMACEUTIQUES NOUVELLES'; TEC & DOC LAVOISIER, PARIS, FR; 1985, pages 467-560, XP002040369 PUISIEUX, F.: "Les liposomes"	
X,P	--- JOURNAL OF CONTROLLED RELEASE, vol. 41, August 1996, AMSTERDAM NL, pages 99-119, XP004037576 ZELPHATI, O. & SZOKA, F.: "Liposomes as a carrier for intracellular delivery of antisense oligonucleotides: a real or magic bullet ?" see page 108, right-hand column, paragraph 7 - page 114, left-hand column, line 17 --- -/--	1,3,4, 14,16, 17,20

INTERNATIONAL SEARCH REPORT

Intern al Application No

PCT/CA 97/00347

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	JOURNAL OF CONTROLLED RELEASE, vol. 41, no. 1/2, August 1996, pages 121-130, XP000592989 BENNETT, C. ET AL.: "PHARMACOKINETICS IN MICE OF A 3H-LABELED PHOSPHOROTHIOATE OLIGONUCLEOTIDE FORMULATED IN THE PRESENCE AND ABSENCE OF A CATIONIC LIPID" see the whole document -----	1,3-5, 14, 16-18, 20,21

INTERNATIONAL SEARCH REPORT

national application No.

PCT/CA 97/ 00347

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 14-22 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 97/00347

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9405333 A	17-03-94	US 5591623 A	07-01-97
		US 5514788 A	07-05-96
		AU 673193 B	31-10-96
		AU 4840193 A	29-03-94
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		EP 0662003 A	12-07-95
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		NO 950800 A	24-04-95
		NZ 256171 A	20-12-96
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		EP 0783296 A	16-07-97
WO 9422468 A	13-10-94	US 5641508 A	24-06-97
		AU 6554594 A	24-10-94
		CA 2159626 A	13-10-94
		EP 0692972 A	24-01-96
		JP 8511510 T	03-12-96